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MECHANISMS OF NUTRITION BAR HARDENING: EFFECT OF HYDROLYZED WHEY PROTEIN AND CARBOHYDRATE SOURCE

by

Shaun P. Adams

A thesis submitted in partial fulfillment
of the requirements for the degree

of

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ABSTRACT

Mechanisms Behind Nutrition Bar Hardening: Effect of Hydrolyzed Whey Protein and Carbohydrate Source

by

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Utah State University, 2008

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The influence of increasing hydrolyzed protein content on the microstructure and hardness of high protein nutrition bars was investigated to determine the mechanism of hardening during storage. Bars with various hydrolyzed protein levels were manufactured using differing ratios of 0, 25, 50, 75, 100% (wt. /wt.) of partially hydrolyzed whey protein isolate (HWPI) to an intact (non-hydrolyzed) whey protein isolate (WPI) which made up approximately 38% of the total bar composition. High fructose corn syrup (HFCS) (42%) and vegetable shortening (20%) constituted the rest of the ingredients. Accelerated aging was performed by storing bars at 32 ºC for 36 d with analysis being performed every 7 d starting at d 2. Hardness was measured as the peak force to penetrate into the bars 8.5 mm using a 45º chisel blade. Microstructure was examined using confocal scanning microscopy with staining for protein and fat. The level of HWPI affected both hardness and microstructure of the bars. Bars that developed the most hardness, with hardness values of 87.6 x 10^2 g-force and 97.2 x 10^2 g-

force, were those that had no added HWPI or only 25% HWPI ($P < 0.05$). Bars with 100% of the protein as HWPI were softest with a value of 24.6×10^2 g-force ($P < 0.05$) and these bars had a microstructure showing a two-phase separation of fat from the aqueous phase containing protein and sugars. The bars that exhibited severe bar hardening had a three-phase separation of the fat, protein, and sugar. The gradual separation of the protein from the sugars into two distinct phases is proposed as the mechanism causing hardening in high protein nutrition bars.

The influence of different carbohydrate sources on water activity, Maillard browning, hardness, and microstructure was then investigated. Bars were formulated using either WPI or HWPI with either 70% HFCS or 70% sorbitol syrup as carbohydrate source. This resulted in four bar types, which were then aged at an accelerated rate through storage at 32 ºC and analyzed again every 7 d. Color and water activity were measured as well as hardness and the microstructure was again observed using confocal microscopy. Changing the carbohydrate component of the bars from HFCS to sorbitol syrup had a large effect on the amount of Maillard browning, no effect on the a_w, and a slight effect on bar hardening and microstructure while using HWPI instead of WPI had a slight effect on browning, an effect on water activity, and a large effect on bar hardening and microstructure. The carbohydrate effect on bar hardening was not to the same degree as using HWPI. Using sorbitol with WPI reduced hardness after 35 d at 32 ºC by 25% while replacing WPI with HWPI reduced hardness by 55%. When using HWPI both the HFCS and sorbitol, bars remained soft (i.e. hardness <500 g-force) through d 27, with the HFCS increasing in hardness ($P < 0.05$) by d 35.

(83 pages)

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LIST OF ABBREVIATIONS

a_w = Water activity

CLSM = Confocal Laser Scanning Microscopy

DSC = Differential Scanning Calorimetry

FITC = Fluorescein isothiocyanate

HFCS = High Fructose Corn Syrup

HPN = High Protein Nutrition

HWPI = Partially Hydrolyzed Whey Protein Isolate

PLM = Polarized Light Microscopy

WPC = Whey Protein Concentrate

WPI = Whey Protein Isolate

XRD = X-ray Powder Diffraction

CHAPTER 1

GENERAL INTRODUCTION

In foods, texture has a significant effect on the acceptance of a product by consumers. Texture perception is an important factor in consumer sensory appreciation (Wilkinson and others 2000), but it is difficult to control due to the many different factors inherent in food that affect its final texture. This is especially true in the whey protein bar industry where the texture of high protein nutrition (HPN) bars goes very rigid within 6 m of manufacture when stored at room temperature, causing consumer avoidance. Initially, this was not a large enough drawback to discourage consumers from buying HPN bars, but as the market has grown and expanded, consumers have begun to expect better products. This has led consumers to avoid purchasing HPN bars that harden quickly during storage and has forced manufacturers to expend resources to fix the problem. However, manufacturers have little knowledge about either the mechanism behind bar hardening or the changes that occur during storage which makes overcoming this problem difficult. Thus, it is necessary to gain a better grasp of what is taking place as the HPN bars harden in order to give the bar industry more options in dealing with this issue.

LITERATURE REVIEW

Nutrition Bar Industry

The nutrition bar industry encompasses a market of over $3 billion in the U.S. (Halliday 2005). This industry is still growing rapidly and bar manufacturers have many obstacles they need to overcome. One of the main obstacles in the industry is the

decrease of bar acceptability, as the bars age, due to bar hardening (Stefan 2003). Immediately after manufacture most nutritional bars have a soft nougat-like texture, but after one month storage at room temperature the bars harden and after four to six months the bars become hard enough to make chewing difficult (i.e. >3000 g-force hardness). This decline in texture occurs much faster than the deterioration of microbiological quality and results in bars that are unappealing to consumers (Stefan 2003). There is virtually no published scientific work on the changes that take place in HPN bars as they harden, although various manufacturers have developed practical methods that will keep bars soft (Gottschalk 2006). Most involve replacing a portion of the protein with a hydrolyzed protein.

Such methods for keeping HPN bars soft have remained proprietary with little information being released in the public domain. This includes work on the characteristics of hydrolyzed proteins that allow them to keep HPN bars soft. However hydrolyzed proteins may not be the optimum solution to prevent bar hardening because they have other inherent drawbacks, including higher cost, and quality issues such as possible bitter off-flavors and negative textural changes. Hydrolyzed protein is also harder to use in bars because if over-mixed the hydrolyzed proteins tend to lose their softening effect (Paulsen 2008, personal communication). Bars containing hydrolyzed protein also tend to stick to equipment making them harder to process. To find a superior solution to the bar hardening problem, more information is needed on the mechanisms underlying bar hardening, including why hydrolyzed protein retards hardening of HPN bars during storage.

Whey Protein Nutrition Bar Composition

Most whey protein-based HPN bars are made mainly of protein, fat, carbohydrates and water, along with a few minor components including the flavors, stabilizers, and inclusions such as peanuts and fruit. The protein source is usually either a whey protein isolate (WPI) containing >90% protein, a whey protein concentrate (WPC) containing 34 to 80% protein, or a protein blend containing hydrolyzed whey protein. The fat source most often used is either vegetable shortening, cocoa butter or some type of oil (canola or vegetable). A blend of high fructose corn syrup (HFCS) and a sugar alcohol (sorbitol or maltitol) syrup is usually the carbohydrate source. This is usually the only source of water in the bar formulation and this syrup (70 to 80% solids) acts to hold all the ingredients in the bar together.

High protein bar components are typically mixed in 30:30:40 ratios of fat, protein powder, and sugar syrup respectively (Paulsen 2008, personal communication), and initially result in a dough that is soft, malleable, and easily formed into bars. The onset of hardening in HPN bars begins fairly soon after production, however and bars become hard, rigid, and difficult to chew within six months of storage. Even though the complexity of HPN bars make it difficult to pinpoint the actual causes of bar hardening bar manufacturers have come up with numerous products that are designed to keep bars soft over time such as milk protein powders (Stefan 2003), high water activity peptide containing powders (Gautam and others 2006), and hydrolyzed protein containing powders (Gerdes 2005). There is no published scientific data on the mechanisms behind the success of these solutions.

Texture in Bars

To sell a successful food product its texture has to be what the consumer experts want and consider desirable (or at least acceptable) for that particular food. The texture factor of concern in HPN bars is bar hardening, where the protein bar matrix hardens as it ages (Stefan 2003; Gautam and others 2006). This problem intensifies as higher concentrations of protein are added to the bar (Paulsen 2008, personal communication). Texture can be affected by many different elements within the bar, including extrinsic factors such as temperature and humidity (Wilkinson and others 2000) and intrinsic factors such as the structure and properties of the ingredients in the bar and how those ingredients interact with each other (Aguilera and Stanley 1999).

Bar Microstructure

Texture in food can also be related to the microstructure of the food. Understanding microstructural changes that occur during processing and/or storage of food can help in identifying the role of different ingredients and enables better control of the structure and, ultimately, the manipulation of texture (Wilkinson and others 2000). However, there have been few previously reported studies of the microstructure of HPN bars. Knowledge of changes in their microstructure during storage could help in determining the factors which contribute to the hardening of the bar.

Confocal Microscopy

Confocal laser scanning microscopy (CLSM) can be used to image food microstructure with minimal sample preparation (Sheppard and Shotton 1997). This is possible because CLSM does not transmit light all the way through the sample. CLSM uses a focused scanning laser to illuminate a sub-surface section of the sample (Brooker

1995). This allows the light to be reflected off of the sample and passed through a pinhole that blocks any out-of-focus light to yield a thin and sharply focused section of the sample (Brooker 1995). It is this ability that makes CLSM the best technique for imaging the relatively thick samples such as nutrition bars. In order to make the CLSM method more powerful it is often used in conjunction with component specific (protein, lipid, carbohydrate) fluorescent dyes that, when excited at certain wavelengths, fluoresce at longer wavelengths (Invitrogen 2007) . This permits the labeling of separate components in the food system (e.g., fat and protein) and gives us a good idea of how they are oriented in relation to each other.

Proposed Causes of Bar Hardening

There have been a number of mechanisms suggested for causing hardening of HPN bars. One of the proposed causes of bar hardening is the phase transition of the sugars, whether through crystallization or a glass transition. Either transition would cause the sugar to lose its ability to hold water. The water originally bound by the sugar then migrates into the protein portion of the bars causing over-hydration and possibly a conformation change in the protein. This may occur due to the high concentrations of sugars that are present in bars making crystallization possible. In high protein nutrition bars the concentration of the sugars used ranges from 70% to 76% solids. This concentration in the original syrup is just below the concentration required to force the sugar into a glass transition (Hartel 2001) instead of crystallization before it is mixed with the proteins. If the proteins then pull water away from the sugars as they become partially hydrated through mixing the sugar concentration would increase. This increase coupled with the glass transition temperatures of 10 °C and 36 °C for fructose and

glucose of respectively (Hartel 2001) may make a glass more likely to form as the temperatures are close enough to room temperature that a mixture of glucose and fructose such as high fructose corn syrup might be expected to have a glass transition temperature of around 23 °C. Other proposed causes derived from changes in proteins are the formation of disulfide bonds (Zhou and Labuza 2008). Disulfide bonding was proposed based on studies using an aqueous phosphate buffer system with whey protein in which water comprised 40% of the formulation. This level may be too high to appropriately model an actual HPN bars containing only 10 to 15% moisture. Maillard browning is another protein based reaction involving protein cross-linking that may also be involved. Any protein cross-linking based interaction would result in a more rigid structure possibly causing bar hardening.

Phase Separation in Foods

Phase separation is a major determinant of a food's texture, mechanical stability, consistency and, ultimately, appearance and taste (Semenova 2007). Rogers and others (2006) reported that phase separation can result from the presence of multiple polymers in solution, which reduces the number of possible configurations and decreases the chance that one polymer will fit into the domain of the other. In nutrition bars, the proteins are the only polymers present as there are usually no large molecular weight polysaccharides. Suihko and others (2005) reported that phase separation can occur in systems containing simple sugars and proteins because of sugar crystallization when under high stress such as high humidity or temperature. Whether this can occur in HPN bars is unknown, although HPN bars harden more rapidly as storage temperatures increase so it may be the case.

PRELIMINARY RESEARCH

Introduction

One of the proposed causes of nutrition bar hardening is a phase transition of the sugar. This preliminary research was performed to test the validity of the proposed theories that crystallization or a glass transition of the sugar was the underlying causes of bar hardening. Three methods were used to test for the presence of crystals. These methods were polarized light microscopy, X-ray powder diffraction, and differential scanning calorimetry.

Polarized Light Microscopy

Polarized light microscopy (PLM) allows one to distinguish crystals from noncrystalline material (Mazzobre and others 2003). For this reason it was chosen to examine slices of nutrition bar for evidence of crystallization. In order to observe the presence of crystals in the bar matrix small slices (6x6x1 mm) were cut out of the middle of the bar sample. These slices were placed on a microscope slide and viewed. Upon study of the images acquired through this method no crystals were observed. This method yielded inconclusive results as to the presence of crystals however, as it was difficult to acquire sharp clear images because of the inherent limitations of light microscopy such as the large depth of field which allows information from different focal planes to reach the camera (Brooker 1995). This is a drawback in the study of HPN bars because it is difficult to maintain the microstructure if a sample must be smeared onto the slide. This may make PLM less than ideal for analyzing nutrition bars.

X-Ray Powder Diffraction

X-ray powder diffraction (XRD) is a powerful technique that can detect even small crystalline regions that exist within complex matrices (Manzacco and others 2002) which would be an apt description of most food systems. X-ray powder diffraction passes X-rays through the material of interest and if crystals are present they will diffract the X-ray. This change forces the X-ray to hit the detector at a different angle and it can be used to calculate the approximate size, shape of the crystal, and the molecular orientation in the crystal. The method followed by Suikho and others (2005) was used to look for any possible crystals within the bar matrix. Again no evidence of crystals was found within the bar matrix and the X-ray diffractometer yielded a smooth diffraction curve that would be expected from an amorphous system with none of the peaks that would suggest the presence of crystals. This would seem to suggest that the sugar in the nutrition bars is not crystallizing, but may be forming a glass or remaining a solution.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is used to give a great amount of information about thermal transitions of food materials (Roos 1987). In DSC studies a reference (usually an empty pan) is used which is heated or cooled at a set rate. The amount of energy used to heat or cool the reference is then compared to the amount of energy needed to heat the sample at the same rate (Hohne et al 2003). In this way the presence of crystals or a glass can be detected as they are both undergo endothermic reactions when changing from solid state to liquid and a glass transition will cause an endothermic shift in the baseline heat capacity. The nutrition bar samples were analyzed by DSC by taking a 10 to 15 mg sample from the middle of the bars and placing it into a

hermetically sealed pan. The sample was then heated from room temperature (23 °C) to 100 °C at a rate of 5 °C/min and the thermogram was studied looking for any endothermic transition. The only transition that was found on all thermograms was a small dip at around 50 °C which would coincide with the melting point of the shortening used in the bar. There was a glass transition-like dip on one bar sample analyzed at d 22, but it could not be repeated and no other bars showed a similar peak. This data suggests that the sugar in the bar is not undergoing a transition such as crystallization or glass formation. In light of this preliminary data it was decided to look at the actual bar microstructure in conjunction with texture analysis testing for hardness.

HYPOTHESIS AND OBJECTIVES

I hypothesize that the increases in hardness of whey protein-based HPN bars during storage are related to a change in the structure of the bar matrix. The aim of this work is to gain a greater understanding of the changes that take place within HPN bars as they harden over time. The specific objectives are:

(1) Determine the effect of hydrolyzed protein concentration on the hardness and microstructure of high protein nutrition bars.

(2) Determine the effect of carbohydrate source on the hardness and microstructure of high protein nutrition bars.

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CHAPTER 2

HARDENING OF HIGH PROTEIN NUTRITION BARS AND SUGAR-PROTEIN PHASE SEPARATION

ABSTRACT

The objectives of this study were to determine how hydrolyzed proteins affect the hardness of whey protein nutrition bars and what changes take place during the hardening process. Bars were formulated using ratios of 0, 25, 50, 75, or 100% (wt./wt.) partially hydrolyzed whey protein isolate/whey protein isolate blend (HWPI) to an intact (non-hydrolyzed) whey protein isolate (WPI) making up approximately 38% of total bar composition. High fructose corn syrup (42%) and vegetable shortening (20%) constituted the rest of the ingredients. These ingredients were mixed until a soft nougat-like dough was formed. These doughs were then extruded into bars measuring 6.5 x 3.0 x 0.1 cm. After sealing in foil-lined packaging film the bars were stored at 32 °C for 36 d of accelerated shelf life testing, and analyzed every 7 d. Hardness was measured as peak load during penetration by a 45° steel wedge, and microstructure was observed using confocal laser scanning microscopy with protein and fat staining.

Bars initially had similar hardness of $3.5 \pm 0.4 \times 10^2$ g-force and then gradually had increased hardness. The more HWPI in the bar formula the slower their rate of hardening, bars containing 100% and 0% HWPI had mean hardness of 24.6×10^2 g-force and 87.6×10^2 g-force, respectively. There was no hardness reduction in bars containing 25% which also required longer mixing times than other HWPI containing bars, whereas bars containing 50% HWPI needed no more mixing, than those bars containing 75 and 100% HWPI, and showed a reduction in hardness. Bar microstructure at d 2 showed

protein and fat dispersed in particulate form throughout the carbohydrate syrup within the bar matrix. During storage, bars made with the higher levels of hydrolyzed protein (75 and 100% HWPI), exhibited separation of fat from the aqueous phase containing protein and carbohydrate. In the bars containing only 0 or 25% HWPI a separation of the sugar syrup from the protein into a distinct phase was observed. We propose that the bar hardening that limits the shelf life of high protein nutrition bars is a function of a microscopic phase separation between the protein and sugar syrup.

INTRODUCTION

In foods, texture has a significant effect on the acceptance of a product by consumers because texture perception is an important part of consumer sensory appreciation (Wilkinson and others 2000). This is especially true in the whey protein bar industry where high protein nutrition (HPN) bars go rigid much faster than any deterioration of microbiological quality occurs which results in bars that are unappealing to consumers. When HPN bars first started being sold this was not a large enough drawback to discourage consumers from buying, but consumers have come to expect better products as the food bar market has grown to encompass $3 billion (Halliday 2005). This industry continues to mature, and the consumer avoidance of HPN bars that harden quickly during storage has led manufacturers to expend resources in an effort to fix the problem (Stefan 2003).

In general high protein bars consist mainly of protein, fat, and carbohydrates, with a few minor components including flavors, stabilizers, and inclusions such as peanuts and dried fruit. Whey proteins are often used as the protein source as either whey protein isolates (WPI) or a 70 to 80% whey protein concentrates (WPC). Sometimes hydrolyzed

proteins are added to improve bar texture (Gottschalk 2006). The fat sources most often used are vegetable shortening, cocoa butter or vegetable oil. A blend of high fructose corn syrup (HFCS) and a sugar alcohol (sorbitol or maltitol) is usually the carbohydrate source which is in a syrup form and acts to hold the other bar components together. High protein bar components are mixed in approximately 30:30:40 ratios respectively and initially result in a dough that is soft, malleable, and easily formed into bars. The onset of hardening in such HPN bars begins fairly soon after production and the bars tend to become hard and unstable over time resulting in a shelf life of less than 6 mo at room temperature (Gautam and others 2006).

For the last 10 y the protein industry has been working hard to overcome this bar hardness problem. By including a portion of hydrolyzed protein in the bar formulations the bars maintain softness for longer but the mechanism that allows hydrolyzed protein to decrease bar hardness is unknown. Some of the suggested mechanisms include sugar crystallization, glass formation, Maillard browning, disulfide crosslinking, or dehydration, but to date there has been no published scientific studies of this phenomenon as it occurs in these bars.

While hydrolyzed proteins provide a practical solution, they also have their own quality issues such as bitter off flavors, different mouthfeel, and other textural changes. In order to find a superior solution to the bar hardening problem, the actual mechanism causing bar hardening needs to be determined in order to understand why hydrolyzed protein decreases hardening during storage. This study was carried out with the aim of visualizing the differences in microstructure between bars made with a partially

hydrolyzed whey protein isolate blend and bars made using a WPI, and what those differences suggest.

MATERIALS AND METHODS

Bar Manufacture

Bars were made in 1.2-kg batches with each batch starting with 456 g of protein powder. The protein powders used were a WPI (Provon® 190; Glanbia Nutritionals, Inc., Twin Falls, ID) and HWPI (BarFlex® 191; Glanbia Nutritionals, Inc., Twin Falls, ID) that is a protein powder used to keep bars soft. These were added so as to form protein blends containing 0, 25, 50, 75, and 100% BarFlex. Five-hundred and twelve grams of HFCS and 232 g of Crisco brand vegetable shortening were then added. These ingredients were combined in a 5-qt mixing bowl (Kitchen Aid, St. Joseph, Michigan) and mixed until a smooth nougat-like texture was achieved. The dough was formed into a small cylindrical mass and fed into a bar former (Bepex Hutt, Leingarten, Germany) with no heat which rolled the dough out and cut it into bars. The bars measured roughly 6.5 cm x 3 cm x 0.1cm. The finished bars were packaged in foil-lined pouches (Mylar®, Sorbent Systems, Los Angeles, CA) and labeled for storage.

Bar Storage

Accelerated shelf life testing was used with the packaged bars stored at 32 °C for 29 d. The bars were analyzed at 7 d intervals starting at d 2. Analysis included visual observation of the bars, texture analysis to obtain hardness values, and examination of bar microstructure using confocal laser scanning microscopy (CLSM).

Texture Analysis

Texture analysis was performed using a TA.XT Plus texture analyzer (Texture Technologies, Ramona, CA) with 30-kg load cell, an analysis speed of 1 mm/sec, and an activation force of 5 g-force. This analysis was performed on bar samples approximately 10 mm thick using a TA-42 knife blade with sharp 45^{o} chisel end. Bars were placed on the platform and a hardness measure was taken three times on each bar. Hardness was measured as the peak force required to penetrate 8.5 mm into the bars.

Confocal Microscopy

The CLSM method used was similar to the method used by Libaek and others (2006) with a few modifications. Squares of bar with the dimensions of 8 x 8 x 2 mm were sliced from the middle of the bar at room temperature (≈ 22 °C). The square piece was then placed on a pre-washed microscope slide. One drop of a 0.02% (wt./wt.) fluorescein isothiocyanate (FITC) solution in absolute acetone was applied to the piece and allowed 60 s to penetrate the sample. One drop of a 0.02% Nile Red solution in absolute acetone was then applied and allowed to penetrate for 60 s. The piece was covered with a glass cover slip and sealed with air impermeable gel sealing in the moisture and preventing drying.

An inverted microscope (Biorad, Hercules, CA) with an Ar/Kr laser used to excite the FITC at a wavelength of 488 nm and the Nile Red at 568 nm. This yielded emission with peak wavelengths of 520 nm for the FITC and 640 nm for the Nile Red. The fluorescence was captured sequentially using filters of wavelengths 512 to 532 nm for the FITC and ≥ 585 nm for the Nile Red. Due to time and resource constraints 24 images of the bars containing 0% and 100% HWPI were taken at each week as they were expected

to show the most extreme differences, and only four images were acquired for the 25, 50, and 75% HWPI bars. In the final false colorized images, the fat appeared as red and the protein appeared as green, while areas without protein or fat appear as black. In order to confirm that the black areas of the images were carbohydrate and not air or water the plane of focus was moved up and down within the black areas in an attempt to bring the bottom of the hole into sharp focus.

Image Analysis

Micrographs were further analyzed using AnalySIS (Olympus Soft Imaging Solutions, Lakewood, CO) with arbitrary color values set, with the "set thresholds" function, by looking at one image and inputting red, green, and black values for each color (red, green, or black). Then using the "phase analysis" function of the software those values were applied to all images for the 0, 25, 50, 75 and 100% HWPI bars, and the percent red, green, and black areas in the images were calculated.

Statistical Analysis

Bars were made from duplicate batches of dough. A completely randomized design was used to analyze the data with one factor (HWPI concentration) and two replications per factor (dough batches). A measurement of hardness was taken at six different times, the time factor is nested within the replication. Data were analyzed in SAS 9.1 (SAS Inst. Inc., Cary, NC) with a significance level of $P \leq 0.05$ using the Proc Mixed function, with the protein level and the day as the fixed variables and the batch as the random variable. Differences of least squares means was used to determine significant differences.

RESULTS

Physical Properties

During manufacture of the dough differences were observed in the amount of time needed to mix the ingredients into a dough. The doughs without any HWPI in the formulation did not form as readily and required more mixing time. When HWPI was included, the doughs formed more readily and required less time. With only 25% HWPI it still took longer to mix the ingredients and form the dough which may have influenced the resulting hardness.

During bar extrusion, differences in bar consistency and color were observed. Immediately following manufacture all bars were nougat-like in consistency, but the bars without HWPI in them had a shorter less flexible texture and were white in color. As the level of HWPI increased the bars became more flexible and darker in color. The bars with 75 and 100% HWPI were taffy-like in texture and cream colored. These differences were apparent throughout the 36 d storage period.

During storage all bars also underwent changes in both color and consistency. The 0% HWPI bars were a light tan color after 7 d at 32 °C and were caramel colored after 36 d storage. Their consistency became increasing hard and brittle during storage. Adding HWPI to the dough formulation produced bars that were darker both initially and after 36 d of storage. Bars containing 100% HWPI were caramel in color after 7 d, and after 36 d of 32 °C storage were a dark coffee color. The consistency of these bars remained more flexible and taffy-like throughout storage.

Hardness

Both the storage time and concentration of HWPI significantly influenced the hardness of the bars (see Appendix A Table 7). After manufacture (d 2) all bars had similar hardness (peak force during penetration) of 3.5 ± 0.4 x 10^2 g-force. Within 7 d of 32 °C storage significant differences in bar hardness between bar treatments were observed (Table 1). Bars with 0 and 25% HWPI were much harder than other bars and not significantly different from each other with average hardness values of 16.9 x 10^2 and 17.0 x 10^2 g-force, respectively. Bars with 100% HWPI were the softest with an average value of 5.3 x 10^2 g-force. These trends continued throughout storage (Figure 1). This decrease in hardness with increasing amounts of HWPI was expected because HWPI is used commercially for this purpose.

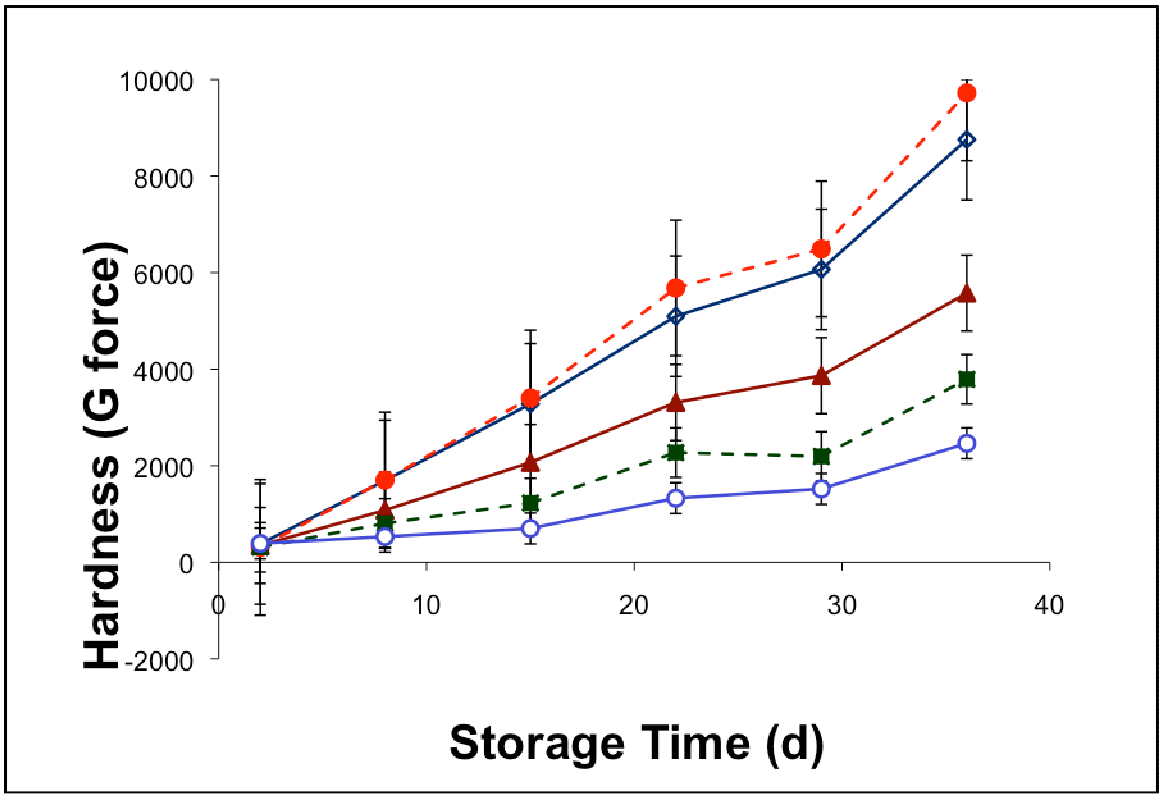

Figure 1. Penetration hardness of high protein (35%) nutrition bars made so that the protein portion consists of 0 (◊), 25 (●), 50 (▲), 75 (■), 100% (○) partially hydrolyzed whey protein isolate over 36 d accelerated storage at 32 °C.

Table 1. Penetration hardness values of high protein (35%) nutrition bars formulated so that the protein portion consists of 0, 25, 50, 75, and 100% partially hydrolyzed whey protein isolate (HWPI) over 36 d accelerated storage at 32 °C**.**

Storage (d)	% HWPI				
	0	25	50	75	100
	10^2 x g-force				
2	3.8^{az}	3.1^{az}	3.5^{az}	3.1^{az}	3.9^{az}
8	16.97^{ay}	17.0^{ay}	10.8^{by}	8.1^{by}	5.3^{by}
15	32.8^{ax}	34.0^{ax}	20.7^{bx}	12.3^{cx}	7.0^{cx}
22	50.9^{aw}	56.8^{aw}	33.1^{bw}	22.7^{cw}	13.3^{cw}
29	60.6^{aw}	64.9^{aw}	38.7^{bw}	21.9^{cw}	15.2^{cw}
36	87.5^{av}	97.2^{av}	55.7^{bv}	37.9^{cv}	24.6^{dv}

a,b,c,d Mean values (n = 6) in same row with no common superscript differ ($P < 0.05$).
v,w,x,y,z Mean values (n = 6) in same column with no common superscript differ ($P < 0.05$).

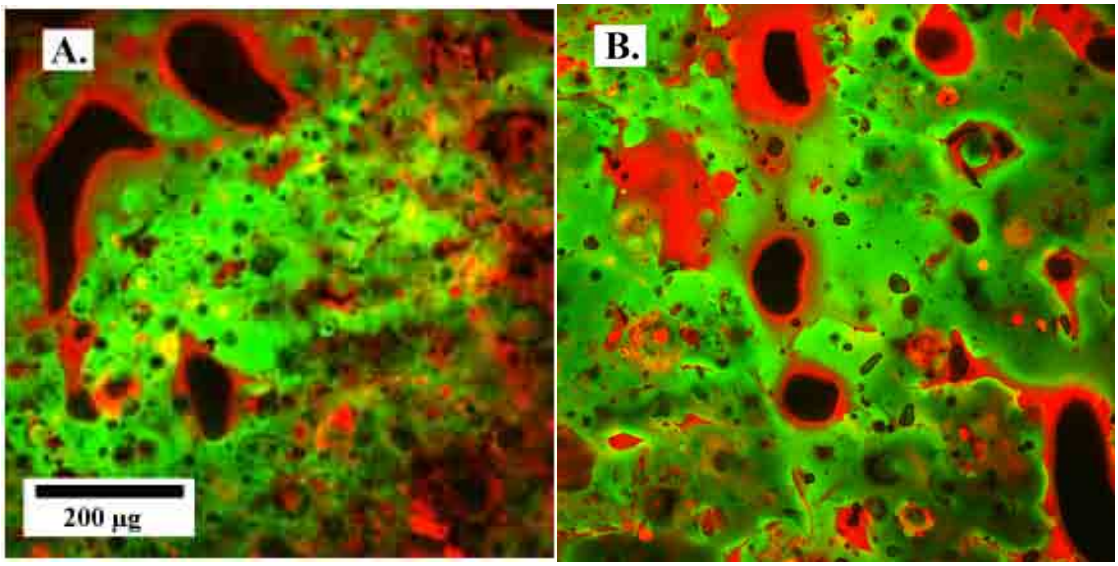

Figure 2. Confocal laser scanning micrographs at d 2 of high protein (35%) nutrition bars formulated using 0% HWPI (A) or 100% HWPI (B) showing protein, fat, and sugar syrup dispersed throughout the bar matrix in particulate form. Green corresponds with dyed protein, red is dyed fat, and black areas are assumed to be the carbohydrate as there is no other un-dyed ingredient in bar.

Microstructure

When observed using CLSM the microstructures of all bars at d 2 were similar. When comparing bars with 0% or 100% HWPI (Figure 2) it was observed that the ingredients were dispersed throughout the bar matrix with some of the protein still in particulate form

and some in the aqueous phase. Very little black, which is assumed to be carbohydrate as it is the only un-dyed ingredient in the bar, was observed in the images. This assumption is supported by the fact that we were unable to bring the bottom of the void into sharp focus as would be possible if it was air or water. The fluorescence was always clouded as it had to pass back through the thicker medium of the carbohydrate syrup (Additional images from all weeks are located in Appendix B). Then by d 29 (Figure 3) a distinct difference was observed between bars made completely with WPI (0% HWPI) and made

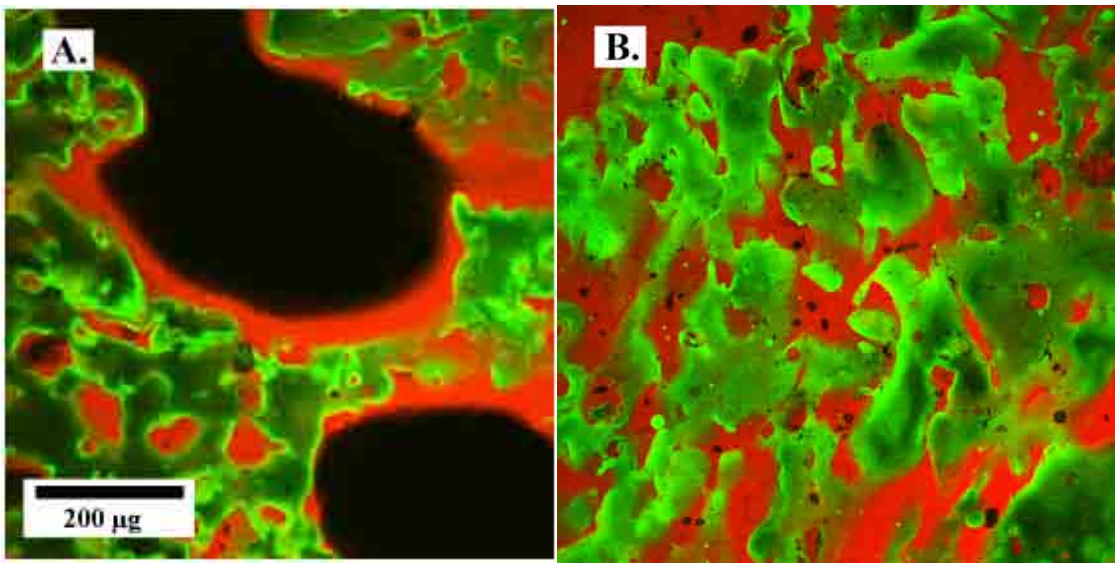

Figure 3. Confocal laser scanning micrographs at d 29 of high protein (35%) nutrition bars formulated using 0% HWPI (A) or 100% HWPI (B) showing a protein, fat, and sugar phase separating into three distinct phases in the 0% HWPI bar and into a fat phase and an aqueous phase containing protein and sugar in the 100% HWPI bar. Green corresponds with dyed protein, red is dyed fat, and black areas are assumed to be the carbohydrate as there is no other un-dyed ingredient in bar.

completely with the partially hydrolyzed whey protein isolate (100% HWPI). Bars containing no HWPI had converted into a three phase system with a distinct separation of fat, protein, and carbohydrate phases. In contrast bars with 100% HWPI only showed a two-phase separation of the fat from a continuous aqueous phase containing both the protein and the carbohydrate. Bars containing 0 and 25% HWPI were not observably different in microstructure with both showing a distinct three-phase separation. Bars containing 50% HWPI showed a moderate three phase separation that was distinctly less extensive than bars containing 0 and 25% HWPI. There was also no observable difference in the microstructure of bars containing 75 and 100% HWPI, with both showing only a two phase separation of the fat phase from the aqueous phase containing

a relatively homogenous mixture of carbohydrate and the protein as indicated by the absence of large areas (black) that lack both protein and fat.

Image analysis showed some distinct differences in those bars containing 0% and 100% HWPI, but statistical analysis gave no significant differences in the intermediate bars which may be due to the fact that only four images were acquired. The amount of protein as well as the storage time significantly affected the microstructure of the bars (see Appendix A Tables 8-10). In the images captured on d 2 of storage, the percentages of each color are very similar regardless of whether the bars have 0% HWPI or 100% HWPI (see Table 2). When comparing bars with 0% or 100% HWPI (Figure 2) it was observed that red made up approximately 20% of all the color in the image, green made up 68%, and black made up 10%. Then by d 8 the microstructure of the 0% HWPI bars showed a slight increase in red (lipid) to 25% of all the color, but the green (protein) decreased to 33% and the black (carbohydrate) increased to 40% as the protein and the carbohydrate started to phase separate. While the microstructure of the 100% HWPI bars showed very little change in red, green, and black percentages with values of 31, 64, and 4% respectively. These trends continued throughout storage with the percentage of fat remaining constant regardless of the amount of HWPI in the bar formulation and the amount of green and black remaining relatively constant as long as both the sugar and the protein remained both hydrated and miscible with each other as in the 100% HWPI bars. A low amount of black in any image was interpreted as the carbohydrate being miscible with the protein and evenly distributed with the protein throughout the mostly green areas of the image. While a high amount of black was interpreted as the carbohydrate and the protein separating from each other into distinct phases. There is a distinct increase in the

amount of black and a decrease in the amount of green in the bars containing just intact WPI and no HWPI. While in the bars containing 100% HWPI the amount of black and green remains fairly constant at about 10% ± 5 and 63 ± 5 throughout all weeks. Image analysis of the microstructure of bars made using 0% and 100% HWPI supports the conclusion drawn from the visual observation of the micrographs, that the ratio of HWPI to WPI has a considerable effect on the bar microstructure.

DISCUSSION

Phase Separation

When nutrition bars are manufactured the ingredients are mixed together forming a dispersion of components consisting of fat droplets and protein particles dispersed in a continuous phase of sugar syrup. The proteins are still only partially hydrated at d 2 due to the low water content (≈15% wt/wt) present in the bars. During storage it appears that the proteins become hydrated and intermix with the sugar forming an aqueous sugar/protein phase. The limited amount of water present would imply that this isn't a true solution. This makes high protein nutrition bars a unique food system having a large amount (35 to 40% (wt/wt)) of highly concentrated (> 80% (wt/wt)) protein powders and sugars syrups. Then during extended storage, the proteins separate from the carbohydrates as shown by the distinct aqueous areas in the protein structure showing both protein (green) and non-protein with no fat (black). Since neither the sugar nor the protein components are soluble in the lipid phase then those areas of the bar structure that fluoresce with the Nile Red stain can be assumed to be fat. However, since both the protein and sugar are water soluble then it is possible they can be present together. Usually in simple sugar-protein systems such phase separation is a result of sugar

Table 2. Percentages of red, green, and black in micrographs of high protein (35%) nutrition bars formulated so that the protein portion consists of 0, 25, 50, 75, and 100% partially hydrolyzed whey protein isolate (HWPI) over 36 d accelerated storage at 32 °C. Red corresponds with dyed fat, green is dyed protein, and black areas are assumed to be the carbohydrate as there is no other un-dyed ingredient in bar.

Storage	% Pixel Color														
Time	0% HWPI			25% HWPI			50% HWPI			75% HWPI			100% HWPI		
(d)	Red	Green	Black	Red	Green	Black	Red	Green	Black	Red	Green	Black	Red	Green	Black
	----------%----------														
2	20^{az}	68^{az}	9^{az}	11^{az}	53^{az}	34^{az}	11^{az}	60^{az}	27^{az}	12^{az}	51^{az}	34^{az}	19^{az}	68^{az}	10^{az}
8	25^{az}	33^{ay}	40^{ay}	6^{bz}	76^{az}	15^{abz}	11^{az}	46^{az}	41^{az}	12^{az}	33^{az}	50^{az}	31^{az}	64^{az}	4^{bz}
15	19^{az}	35^{ay}	40^{ay}	17^{az}	56^{az}	24^{az}	14^{az}	61^{az}	23^{az}	9^{bz}	45^{az}	42^{az}	27^{az}	59^{bz}	8^{bz}
22	16^{az}	44^{ay}	35^{ay}	32^{az}	45^{az}	18^{az}	16^{az}	41^{az}	40^{az}	19^{az}	48^{az}	30^{az}	23^{az}	60^{az}	14^{by}
29	18^{az}	53^{az}	24^{az}	19^{az}	51^{az}	27^{az}	14^{az}	55^{az}	29^{az}	8^{bz}	44^{az}	46^{az}	25^{az}	65^{az}	7^{bz}

a,b,c,Means for the same color within the same row with no common superscripts differ ($P < 0.05$).
x,y,z Means within the same column with no common superscript differ ($P < 0.05$).

crystallization or a glass transition (Suikho and others 2005). In our study, there was no evidence of sugar crystallization or glass transition (unpublished data) when the bars were examined by x-ray crystallography or differential scanning calorimetry. The phase separation in high protein nutrition is unique for sugar-protein systems and thus, may be similar to the type of phase separation that occurs in high concentration protein-polysaccharide solutions. The phase separation between protein-polysaccharide occurs as the dissimilar biopolymers sterically interfere with each other and when mixed together at high concentrations will result in one biopolymer forming the continuous phase and the other being dispersed throughout (Tolstoguzov 1998). In the case of nutrition bars the sugar syrup acts as the continuous phase and the protein is the dispersed phase. One explanation for phase separation is that over time the continuous phase pulls more and more water away from the dispersed phase concentrating it until a high enough concentration is reached and the dispersed phase can no longer remain miscible with the diluted continuous phase (Tolstoguzov 1998). Another explanation is that the steric hindrance / steric compression that occurs when the proteins and sugar are brought together in very high concentrations decreases the entropy of the system making such a dispersion energetically unfavorable. Separation into two phases reduces the steric effect and the gain in entropy makes up for the sugar and protein becoming more concentrated. So rather than being a continuous phase of sugar syrup that provides long term flexibility, the continuous phase is interrupted by a non-flexible partially dehydrated protein phase.

Hydrolyzed Proteins

The observation that immediately after manufacture, all bars regardless of the amount of HWPI in the formula were similar in hardness and microstructure can be attributed to the dry protein particles only being dispersed throughout the bar matrix and the system not having reached an equilibrated state with the proteins being hydrated. Gautam and others (2006) reported that by utilizing high water activity proteins which are more hydrated prior to inclusion in the bar; it is possible to reduce bar hardness. Perhaps the hydrolyzed protein functions by having higher water binding properties than intact proteins (Sinha and others 2007). This would allow them to inhibit the withdrawal of water molecules from the protein by the sugars and retard phase separation. Hydrolyzed proteins might also hinder phase separation because of their smaller molecular weight which would reduce the extent of steric interaction and make them more compatible with the sugar molecules. The increased number of ionizable sites obtained by hydrolyzing the proteins might allow electrostatic interaction between the carbohydrates and the proteins in the bar mixture increasing their compatibility, and the smaller size of a portion of the HWPI may reduce the number of long range interactions and yield softer bars.

Industrial Application

The method of adding hydrolyzed protein to bar formulation has been effective in allowing manufacturers to make bars that will stay soft over the duration of their shelf life (≈ 6 mo at room temperature with 1 y the eventual goal). It is possible that there may be other solutions to the problem of bar hardening other than using hydrolyzed proteins. The limitations when using hydrolyzed proteins are they usually involve higher cost, they

can impart bitter off flavors based on the extent and nature of the hydrolyzed protein, and the can be more difficult to process. Over-mixing of bar doughs containing hydrolyzed proteins can result in an immediate hardening of the bars. The knowledge that adding hydrolyzed proteins to HPNBs inhibits phase separation and prevents bar hardening can be used to overcome bar hardening by looking for other ways to stop phase separation.

CONCLUSIONS

In this experiment we used techniques that would allow us to see the microstructure of the bar with the least amount of disruption and moisture loss. This allowed us to see that HPN bars are a dispersed mixture at manufacture consisting of partially-solid fat droplets and protein powder particles held together with sugar syrup. This mixture has a relatively low amount of available water and the protein powder and the sugar syrup are both at extreme concentrations near 80% (wt/wt). Given the low amount of water the proteins hydrate slowly forming an aqueous phase containing both protein and sugar with a dispersed fat phase and initially all bars regardless of the amount of HWPI had similar hardness and microstructure. After 35 d accelerated storage at 32 °C all bars increased in hardness, but adding HWPI retarded hardening and as the amount of HWPI in the bar increased the hardness decreased with bars containing 100% HWPI remaining soft throughout storage. In the hardest bars (0% HWPI and 25% HWPI) a three phase microstructure was observed by CLSM, with separate fat, protein, and carbohydrate phases while the carbohydrate and protein remained a combined phase in the softer bars. This separation of the protein from the carbohydrate occurred without any apparent crystallization or glass transition of the sugar and is proposed to be the cause of HPN bar hardening.

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CHAPTER 3

EFFECT OF CARBOHYDRATE SOURCE ON THE TEXTURE AND MICROSTRUCTURE OF NUTRITION BARS ANALYZED USING TEXTURE ANALYSIS AND CONFOCAL MICROSCOPY

ABSTRACT

The objectives of this study were to determine if different carbohydrate sources had an effect on the amount of phase separation in high protein nutrition (HPN) bars, whether bar hardening in HPN bars was related to the amount of Maillard Browning, and to determine what changes take place during the hardening process. Bars were formulated using either whey protein isolate (WPI) or a partially hydrolyzed WPI (HWPI) making up approximately 38% of total composition with either 70% high fructose corn syrup (HFCS) or 70% sorbitol as a carbohydrate source making up 43% of the total. This resulted in four bar types being made in duplicate for a total of eight bar doughs. Crisco vegetable oil constituted the other 19% if the formulation. These ingredients were mixed until a soft nougat-like dough was formed for the 100% WPI bars while the doughs containing 100% HWPI were much softer, but could still be extruded. The doughs were then extruded into bars measuring approximately 6.5 x 3.0 x 1.0 cm. After sealing in foil-lined packaging film the bars were stored at 32 ºC for 34 d of accelerated shelf life testing and analyzed every 7 d. Color and water activity (a_w) were measured, and hardness was measured as peak load during penetration by a 45° steel wedge. Microstructure was observed using confocal laser scanning microscopy with protein and fat staining.

Bars containing WPI were very white at d 1while those bars containing HWPI were more cream colored. After 34 d storage, the presence of sorbitol in the bar inhibited the amount of browning in the bar while bars containing HFCS browned considerably were considerably browner than at d 1 regardless of the protein. The WPI bars had similar a_w values of 0.64 at d 1 while the HWPI bars were similar at 0.60. After 34 d the presence of HWPI hindered an increase in a_w while the a_w of bars containing WPI had increased significantly to 0.69. There was no significant effect due to the carbohydrate, but the trend within the same protein type was that bars containing HFCS had a higher a_w. The hardness values for d 1 were approximately 17.0×10^2 g-force for the WPI/HFCS bars and $\approx 24.0 \times 10^2$ g-force when WPI and sorbitol were used. Bars containing HWPI had similar hardness values of $2.25 \pm 0.25 \times 10^2$ g-force at d 1. After 34 d, all bars increased in hardness and bars containing HWPI were much softer than the bars containing WPI. Bars formulated with HFCS were also harder than those containing sorbitol within the same protein type. The hardest bars were those containing WPI and HFCS while the softest bars were those containing HWPI and sorbitol. They had mean hardness values of 7891 g-force and 1071 g-force, respectively. The microstructure, after 34 d storage, of bars formulated with WPI exhibited a three phase separation of protein, carbohydrate, and fat with no noticeable difference between bars containing HFCS or sorbitol. Whereas in the bars containing HWPI there was only a two phase separation of the fat from a combined protein/carbohydrate, but the HWPI bars containing sorbitol had a greater amount of fluorescence in the protein phase than those containing HFCS suggesting the beginnings of a phase separation in the HFCS bars. We propose that the bar hardening that limits the shelf life of high protein nutrition bars is a function of phase

separation between the protein and sugar syrup as well as an increase in a_w. We also propose that bar hardening is not a function of Maillard browning.

INTRODUCTION

High protein nutrition bars are part of the $3 billion food bar market (Halliday 2005) which is rapidly maturing. As this market gets more established consumers are demanding better products which has led to research into some of the obstacles that bar manufacturers are facing. One of these obstacles is the hardening of bars during storage which has led some consumers to avoid bars that go hard within a few months of manufacture. Bar hardening is not a new problem and most bar manufacturers have developed practical bar softening methods, but this research is proprietary and very little publically available research has been performed. Due to the complexity of HPN bars formulations the protein source may not be the only factors effect bar hardness. HPN bars represent a unique food system, in which the concentrations of both the protein and the sugar are very high, and as a result little scientific work has been published on systems even remotely similar. One study (Zhou and Labuza 2007) looked at protein interactions and reported that during bar storage a greater number of disulfide bonds are formed between the proteins. This would form a more rigid bar structure and could be a possible cause of bar hardening. Chapter 2 of this work looked at the effect of hydrolyzed proteins on bar hardness and reported that as more hydrolyzed protein was added to the bars the softer they remained and the softest bars included both intact and hydrolyzed proteins. It was also found that in bars that showed excessive hardening a three phase separation of the main ingredients (fat, protein, and carbohydrates) was observed to occur whereas the softer bars only showed a two phase separation of fat from

the aqueous carbohydrate/protein phase. Maillard browning has also been put forward as a possible cause for bar hardening and the type of carbohydrate would have an effect on the hardness of the bar during storage as a result. Crystallization or glass transition of the sugar has also been proposed as a possible cause of bar hardening. In high protein nutrition bars the concentration of the sugars in the original syrup used ranges from 70% to 76% solids is high enough to crystallize under the right conditions and just below the concentration required to force the sugar into a glass transition (Hartel 2001) so this is a viable theory. Although preliminary research outlined in chapter 1 of this work suggests that the crystallization or a glass transition of the sugar is not the cause of bar hardening as no evidence of either was found in any bars. The purpose of this work is to first determine if changing the type of carbohydrate in the bars would show a relationship between the amount of bar hardness and phase separation. Second, is to determine whether the amount of Maillard browning during storage can be related to bar hardness.

MATERIALS AND METHODS

Bar Manufacture

Bars were made in 1200 g batches each batch was made up of 455 g of protein powder blends supplied by Glanbia Foods Inc. (Twin Falls, ID). The protein powders used were a 90% whey protein isolate (Provon® 190) and a 90% partially hydrolyzed whey protein isolate (BarFlex® 191) which is specially formulated to keep bars soft. The protein blend concentrations were either 100% WPI or 100% HWPI. Five hundred and ten grams of HFCS (High Fructose Corn Syrup 55, ADM, Decatur, IL) or sorbitol (Sorbitol 70% solution, ADM, Decatur, IL) two common carbohydrate syrups that are used in the industry, was added along with 232 g of Crisco brand vegetable shortening.

These ingredients were combined in a 5 quart mixing bowl (Kitchen Aid, St. Joseph, MI) and mixed until a smooth nougat-like texture was achieved. The dough was formed into a small cylindrical mass and fed into a bar former (Bepex Hutt, Leingarten, Germany) with no heat which rolled the dough out and cut it into bars. The bars measured roughly 6.5 cm x 3 cm x 10 mm. The finished bars were packaged in foil lined pouches (Mylar®, Sorbent Systems, Los Angeles, CA) and labeled for storage.

Bar Storage

In order to accelerate that aging process the packaged bars were stored at 32 °C for 34 d and analyzed on d 1, 7, 14, 21, 27, and 34. Analysis included visual observation of the bars, water activity testing, color analysis, texture analysis to obtain hardness values, and examination of bar microstructure using CLSM.

Water Activity Testing

Water activity testing was performed using a Pawkit a_w meter (Decagon Devices Inc., Pullman, WA) and the manufacturer's instructions were followed. The meter was calibrated using 6.0 molal NaCl and 13.41 molal LiCl standards. The standards were individually placed in a sample cup and a reading was taken. Both standards yield a reading of the correct a_w. The water activities of the bar samples were then taken. A large enough sample of bar was placed in a sample cup in order to cover the entire bottom of the cup. The meter was then placed on top of the cup and a reading was taken.

Color Analysis

Color analysis was performed using a Miniscan XE Plus portable colorimeter Model 45/O-S (Hunter Associates Laboratory Inc., Reston, VA.). The method of Vissa

and Cornforth (2006) was followed. The colorimeter was standardized using black and white tiles covered with plastic wrap. The samples were removed from the package and also covered in plastic wrap. Three measurements were then taken at different spots on the sample and the average L*, a*, and b* values were calculated by the instrument. The b* value was the value of import as it indicates a change toward yellow and brown (Thomsen and others 2005).

Texture Analysis

Texture Analysis (TA) was completed using a TA.XT Plus texture analyzer (Texture Technologies, Ramona, CA) with 30 kg load cell, an analysis speed 1 mm/sec, and an activation force of 5 g-force. This analysis was performed on bar samples approximately 10 mm thick using a TA-42 Knife blade with sharp 45^{o} chisel end in order to obtain the most accurate and consistent measurement. Bars were placed on the platform and a hardness measure was taken three times on each bar. Hardness was measured as the peak force reached in grams to cut 8.5 mm deep into the bars.

Confocal Microscopy

The confocal imaging method used was similar to the method used by Libaek and others (2006) with a few modifications. Squares of bar with the dimensions of 8 mm x 8 mm x 2mm thick were sliced from the middle of the bar at room temperature. The square piece was then placed on a pre-washed microscope slide (FisherBrand, Loughborough, Leicestershire, UK). One drop of a 0.02% FITC solution in absolute acetone was applied to the piece and allowed 60 s to penetrate the sample. One drop of a 0.02% Nile Red solution in absolute acetone was then applied and allowed to penetrate for 60 s. The piece was covered with a glass cover slip and sealed with air impermeable gel (Taylor

Lube), sealing in the moisture and preventing drying. The microstructure was observed using confocal laser scanning microscopy (CLSM). The microscope (Biorad) was inverted with an Ar/Kr laser used to excite the FITC at 488 nm and the Nile Red at 568 nm. This yielded emission wavelengths of 520 nm for the FITC and 640 nm for the Nile Red. Images were acquired sequentially, and 12 images were acquired each week for each type of bar. In the final false colorized images, the fat appeared as red and the protein appeared as green, while areas without protein or fat appear as black and were assumed to be carbohydrate.

Image Analysis

Micrographs were further analyzed using AnalySIS (Soft Imaging Systems, Lakewood, CO) with arbitrary color values set, with the "set thresholds" function, by looking at one image and inputting red, green, and blue values for each color (red, green, or black). Then using the "phase analysis" function of the software those values were applied to all images d 34 images for all treatments, and the percent red, green, and black areas in the images were calculated.

Statistical Analysis

Bars were made from duplicate batches of dough. A completely randomized design was used to analyze the data with two factors (HWPI type and carbohydrate type) and two replications per factor (dough batches) for a total of four treatments and eight batches. A measurement of hardness was taken at six different times, the time factor is nested within the replication. Data were analyzed in SAS 9.1 (SAS Inst. Inc., Cary, NC) with a significance level of $P \leq 0.05$ using the Proc Mixed function with protein,

carbohydrate, and time as fixed factors and batch as the random factors. Differences of least squares means was used to determine significant differences.

RESULTS

Visual Observations

As the bar doughs were mixed, differences were observed in the amount of mixing time required to form the ingredients into a dough as well as the color and consistency of the doughs. Doughs containing HWPI formed more readily than those with WPI, required less mixing time, were more cream colored, and more flexible and taffy like. There was very little difference between those doughs containing the same protein source and different carbohydrates (HFCS or sorbitol) however. It was also observed that those doughs containing HWPI were much softer than those containing WPI. The bars with HWPI were very soft, sticky, and did not hold their shape perfectly, while those bars containing WPI were soft, dry, and held their shape well.

During storage all bars underwent changes in color and consistency. The bars containing HWPI were much whiter than those containing HWPI at manufacture, but after 7 d storage the bars containing sorbitol maintained their initial color, while those containing HFCS became darker and browner. After 34 d storage there were visually observable differences between bars containing different carbohydrate sources as well bars containing different proteins (Figure 4). The HWPI containing sorbitol had become only slightly browner, while the HWPI/HFCS bars had become a caramel color. The bars containing HWPI/Sorbitol had darkened only slightly from their color at manufacture as well and were now whiter than the HWPI/HFCS bars while those bars containing HWPI/HFCS had become considerably darker in color after 34 d storage.

Figure 4. Photograph after 34 d storage of high protein (35%) nutrition bars formulated using (A) WPI/HFCS which is a rich caramel color, (B) WPI/Sorbitol which maintained its white color, (C) HWPI/HFCS which is dark brown almost black in color, and (D) HWPI/Sorbitol which darkened only slightly during storage and is still a dark cream color.

Water Activity

Water activity was affected by the type of protein in the bars (See Appendix A Table 11). At d 1 the WPI bars had a mean a_w value of ≈ 0.64 while those containing HWPI had a comparatively lower mean of ≈ 0.60 (Table 3). After 34 d of storage the bars containing HWPI had maintained their a_w close to that of d 1 with a value of 0.62. In contrast the bars containing WPI had a significantly higher a_w of ≈ 0.69. Although there was no significant difference between those bars containing the same protein and different carbohydrate, there was a distinct trend. The bars containing HFCS had consistently higher a_w than those containing sorbitol within the same protein treatment (Figure 5).

Table 3. Water activity values of high protein (35%) nutrition bars formulated with either whey protein isolate (WPI) or partially hydrolyzed whey protein isolate (HWPI) and sweetened with either high fructose corn syrup (HFCS) or sorbitol over 34 d accelerated storage at 32 °C

Storage Time	Water Activity			
(d)	WPI/HFCS	WPI/Sorbitol	HWPI/HFCS	HWPI/Sorbitol
1	0.63^{az}	0.64^{az}	0.60^{bz}	0.59^{bz}
7	0.63^{az}	0.63^{az}	0.60^{bz}	0.60^{bz}
14	0.67^{ay}	0.66^{ay}	0.63^{bz}	0.61^{bz}
21	0.69^{ay}	0.68^{ay}	0.66^{ay}	0.61^{bz}
27	0.68^{ay}	0.66^{ay}	0.66^{ay}	0.62^{bz}
34	0.69^{ay}	0.68^{ay}	0.63^{bz}	0.61^{bz}

a,b, Means within the same row with no common superscript differ ($P < 0.05$)
y,z within the same column with no common superscript differ ($P < 0.05$).

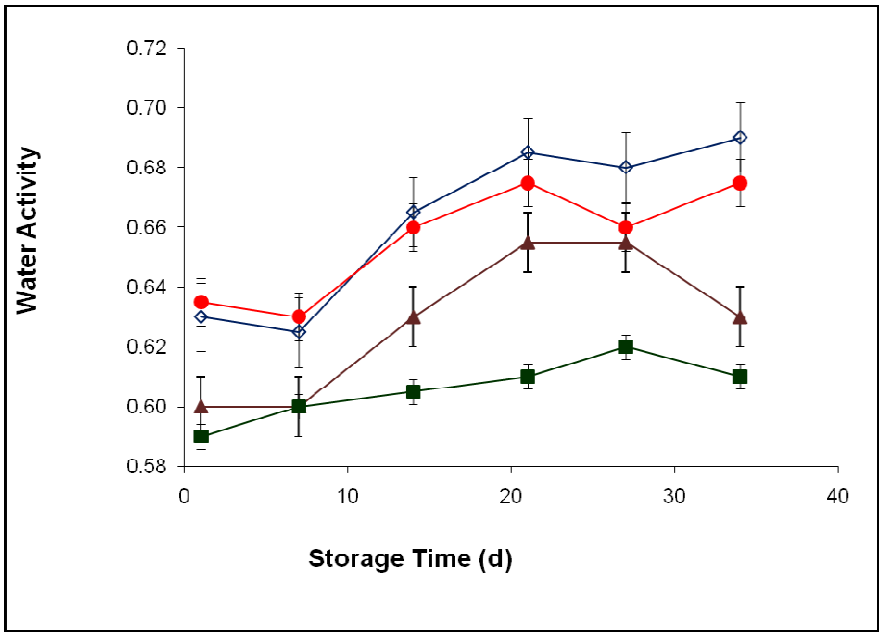

Figure 5. Water activity of high protein (35%) nutrition bars formulated with whey protein isolate and sweetened with high fructose corn syrup (◊) or sorbitol (●), or formulated with partially hydrolyzed whey protein isolate and sweetened with highfructose corn syrup (▲) or sorbitol (■) over 34 d accelerated storage at 32 °C

Color Analysis

Color was significantly affected by the type of carbohydrate used in the bars and slightly affected by the type of protein in the bars. At d 1 the bars containing sorbitol had a smaller b* values (≈12 ± 2) than the bars with the same protein types but containing HFCS (Table 4) and the bar containing HWPI and HFCS was the brownest with a b* value of 22 ± 1. After 34 d of storage the bars containing sorbitol and WPI maintained a b* values very close to the original values at d 1 (14 ± 1) while those bars containing sorbitol and HWPI increased slightly in browness (25 ± 1). Those bars containing HFCS both increased significantly going up to 39 ± 1 and 28 ± 1 for the WPI and HWPI bars respectively. The HWPI bar containing HFCS would have probably been much high, but it may have gotten too dark for the instrument to pick up the b* value and the b* value went down after peaking at d 15 with a value of 41 ± 1 (Figure 6).

Table 4. b* values of high protein (35%) nutrition bars formulated with either whey protein isolate (WPI) or partially hydrolyzed whey protein isolate (HWPI) and sweetened with either high fructose corn syrup (HFCS) or sorbitol over 34 d accelerated storage at 32 °C

	Treatment			
Storage	WPI/HFCS	WPI/Sorbitol	HWPI/HFCS	HWPI/Sorbitol
(d)	b* Value			
1	14.2^{az}	10.0^{bz}	21.6^{cz}	13.8^{az}
7	24.9^{ay}	12.2^{az}	36.6^{by}	16.6^{bz}
14	32.8^{ax}	13.4^{az}	40.5^{by}	18.5^{bz}
21	34.5^{ax}	14.2^{az}	32.7^{ay}	20.1^{by}
27	36.0^{ax}	14.6^{ay}	31.4^{az}	22.4^{by}
34	39.0^{aw}	14.4^{ay}	28.1^{bz}	25.3^{bx}

a,b,c Means within the same row with no common superscript differ ($P < 0.05$).
x,y,z Means within the same column with no common superscript differ ($P < 0.05$).

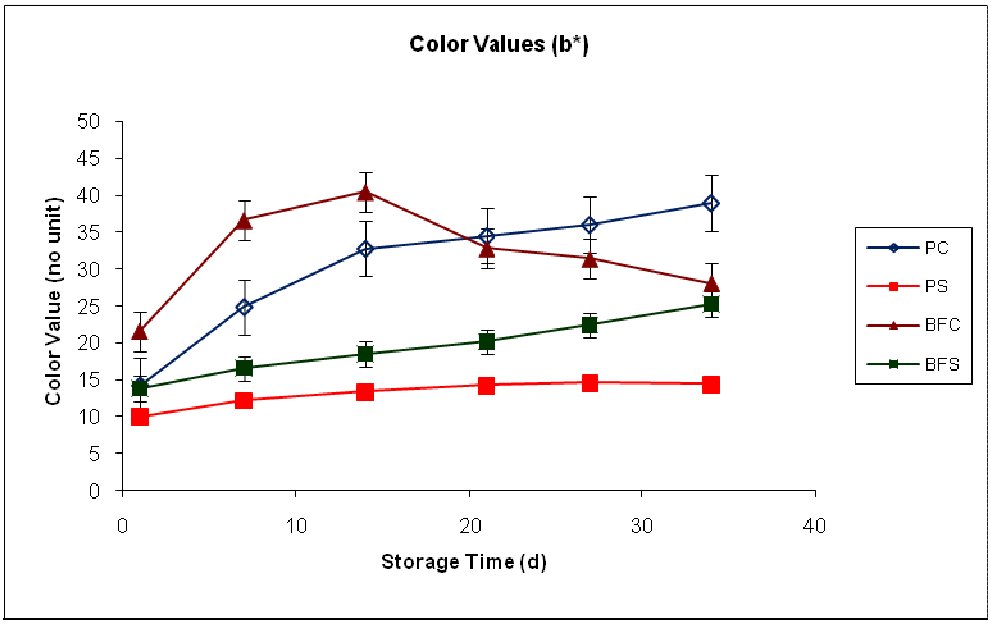

Figure 6. b* values of high protein (35%) nutrition bars formulated with whey protein isolate and sweetened with high fructose corn syrup (◊) or sorbitol (●), or formulated with partially hydrolyzed whey protein isolate and sweetened with highfructose corn syrup (▲) or sorbitol (■) over 34 d accelerated storage at 32 °C

Hardness

Both the storage time and the addition of HWPI had a considerable influence on the hardness of the bars while the type of carbohydrate used in the formulation influences the hardness only slightly (see Appendix A Table 15). At manufacture (d 1) all bars containing HWPI had similar hardness values (peak force during penetration) of 2.3 ± 0.6 x 10^2 g-force (Table 5) while the bars containing WPI were much harder and those containing WPI/sorbitol were the hardest at 24.2 x 10^2 g-force. After 7 d storage at 32 °C significant differences in bar hardness were observed between bars containing different carbohydrate and WPI. Bars formulated using HFCS became significantly harder than those formulated using sorbitol with values of 50.3 x 10^2 and 29.9 x 10^2 g-force respectively. This difference at d 7 was only observed in bars formulated with WPI however, and the bars containing HWPI and HFCS were only slightly harder than those containing HWPI and sorbitol with values of 4.5 x 10^2 and 2.6 x 10^2 g-force respectively. At d 34 all bars with WPI remained significantly harder than those containing HWPI while bars containing HFCS were significantly harder than those containing sorbitol within the same protein type (Figure 7).

Bar Microstructure

When comparing micrographs taken at d 34 distinct differences were observed between those bars formulated using WPI and HWPI as was expected due to the results shown in Chapter 2 (see Figure 2). The microstructure of the WPI bars showed a three component phase separation of fat, protein, and carbohydrate with large black area when either HFCS (Figure 8) or sorbitol (Figure 9) was used as the carbohydrate.

Table 5. Penetration hardness values of high protein (35%) nutrition bars formulated with either whey protein isolate (WPI) or partially hydrolyzed whey protein isolate (HWPI) and sweetened with either high fructose corn syrup (HFCS) or sorbitol over 34 d accelerated storage at 32 °C

	Treatment			
Storage (d)	HWPI/HFCS	HWPI/Sorbitol	WPI/HFCS	WPI/Sorbitol
	G force x 10^2			
1	2.5^{az}	2.0^{az}	16.8^{bz}	24.2^{bz}
7	4.5^{az}	2.6^{az}	50.3^{by}	29.9^{cz}
14	3.8^{az}	1.8^{az}	49.9^{by}	25.2^{cz}
21	5.5^{az}	3.6^{az}	58.1^{by}	41.6^{cy}
27	5.4^{az}	3.5^{az}	54.3^{by}	29.6^{cz}
34	35.6^{ay}	10.7^{by}	78.9^{cx}	57.9^{dx}

a,b,c Means within the same row with no common superscript differ ($P < 0.05$).
z,y,x Means within the same column with no common superscript differ ($P < 0.05$).

In contrast, those bars made using HWPI only showed a two phase separation of the fat from the continuous phase (protein and carbohydrate) and much smaller amount of black (Figures 10 and 11). There were small differences observed between those bars containing different sugars as well. These differences were only noticeable in those bars formulated with HWPI however and the type of carbohydrate used in the WPI bars had no effect. The HWPI bars containing sorbitol showed a lot of bright green fluorescence throughout the continuous phase and very little black. In comparison those bars containing HFCS had a lot of dark areas throughout the protein/carbohydrate phase and were only bright green around the edges. This would seem to suggest that the bars containing HFCS are beginning to phase separate as they reach 34 d of storage time and more un-dyed carbohydrate, that is not miscible with the protein, is becoming visible.

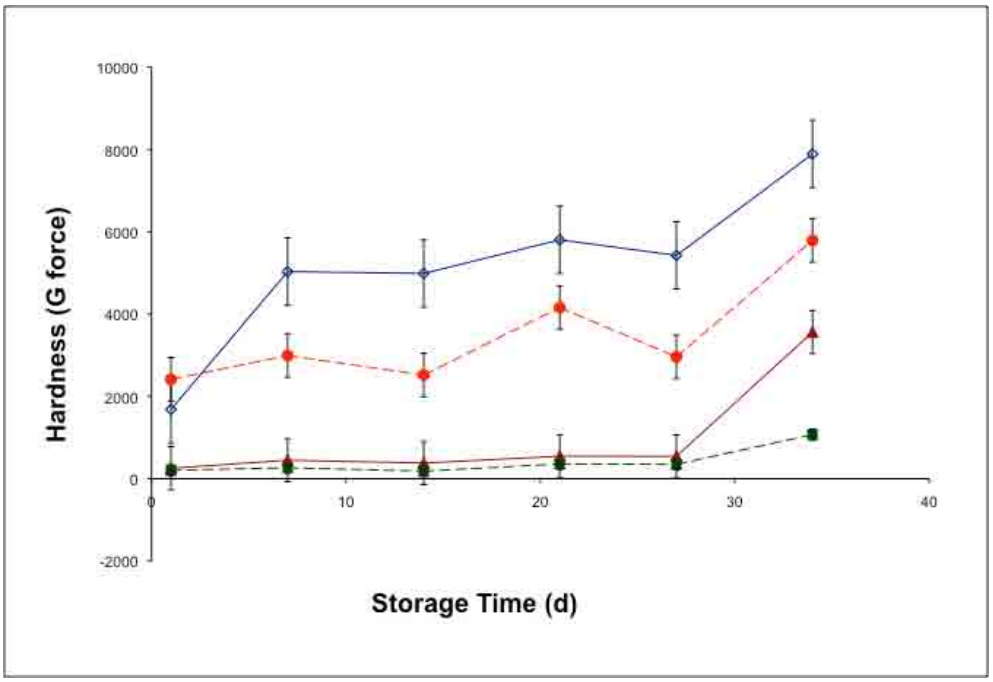

Figure 7. Penetration hardness of high protein (35%) nutrition bars formulated with whey protein isolate and sweetened with high fructose corn syrup (◊) or sorbitol (●), or formulated with partially hydrolyzed whey protein isolate and sweetened with high fructose corn syrup (▲) or sorbitol (■) over 34 d accelerated storage at 32 °C

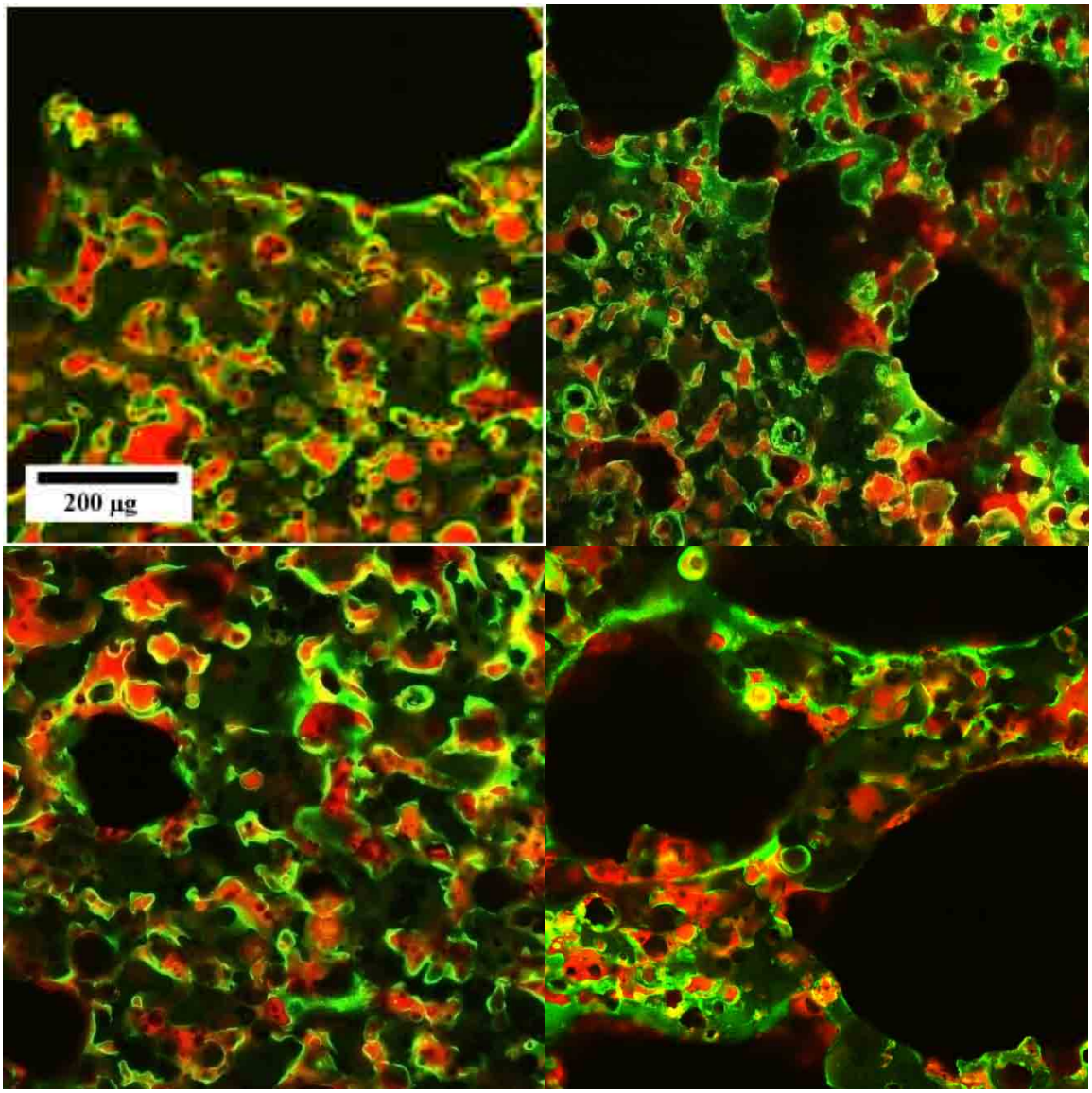

Figure 8. Confocal laser scanning micrographs at d 34 of a high protein (35%) nutrition bar formulated using WPI with HFCS as a carbohydrate source showing a fat, protein, and sugar phase separating into three distinct phases with a great amount of black within the protein matrix. Green corresponds with dyed protein, red is dyed fat, and black areas are assumed to be the carbohydrate as there is no other un-dyed ingredient in bar. The dark areas within the green protein matrix may suggest more sugar separating away from the protein phase.

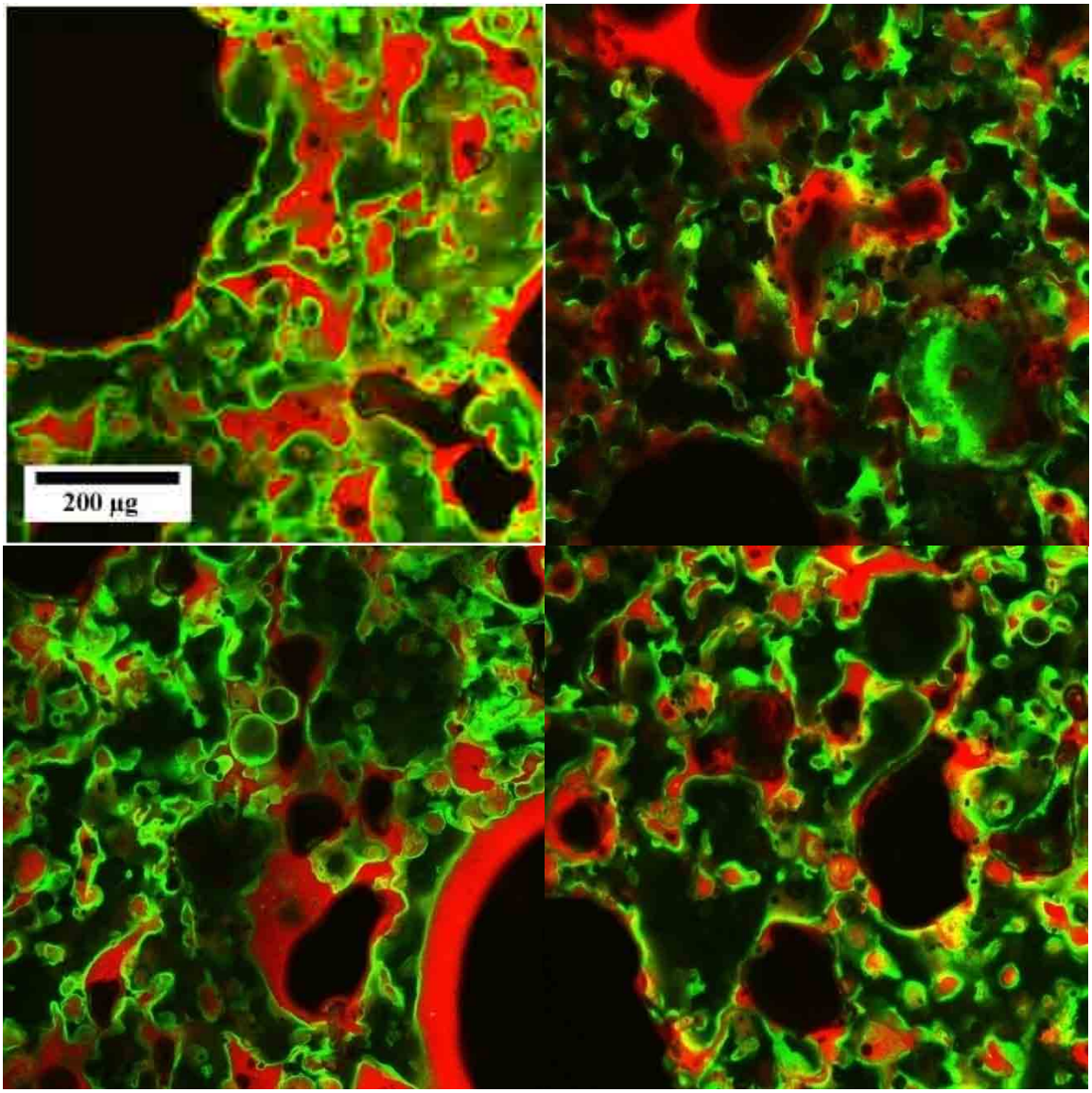

Figure 9. Confocal laser scanning micrographs at d 34 of a high protein (35%) nutrition bar formulated using WPI with sorbitol as a carbohydrate source showing a fat, protein, and sugar phase separating into three distinct phases with a small amount of black within the protein phase. Green corresponds with dyed protein, red is dyed fat, and black areas are assumed to be the carbohydrate as there is no other un-dyed ingredient in bar.

In contrast the sorbitol seems to remain miscible with the protein phase of the bar even after 34 d of storage. In those bars containing WPI the effect from the carbohydrate type seems to be overshadowed as the system is already phase separated after 34 d storage.

Image analysis data (Table 6) shows that the protein has a large effect on the percentage of red green and black within the micro-images as well as the magnitude of the effect of the carbohydrate (see Appendix A Tables 16-18). Distinct trends were present which supported those conclusions drawn from the visual study of the micrographs. Differences in the percentages of red (fat), green (protein), and black (carbohydrate) were observed in the images captured on d 34. The images from the WPI bars were very similar regardless of the carbohydrate type with red, green, and black percentages of $\approx 6 \pm 0.5\%$, $\approx 29 \pm 1\%$, and $\approx 62 \pm 1\%$, respectively. The elevated percentage of black suggests a great amount of carbohydrate has phase separated from the protein. In contrast images of the bars containing HWPI had much more red and green and much less black. The relatively low amount of black in these bars indicates that only a small amount of carbohydrate separated from the proteins. Without the high degree of phase separation between the carbohydrates and the proteins, as seen in the WPI bars, an effect due to the type of carbohydrate was detected in the HWPI bars as well. Those bars containing HFCS had greater percentage of black as the proteins and carbohydrates began to phase separate while those containing sorbitol had less black, as the sorbitol acted to inhibit the separation of the protein and carbohydrate phases. That the effect of the type of carbohydrate was only observable in the bars containing HWPI suggests that as the degree of phase separation increases in the bar the effect of carbohydrate type diminishes as it is not able to inhibit phase separation on its own.

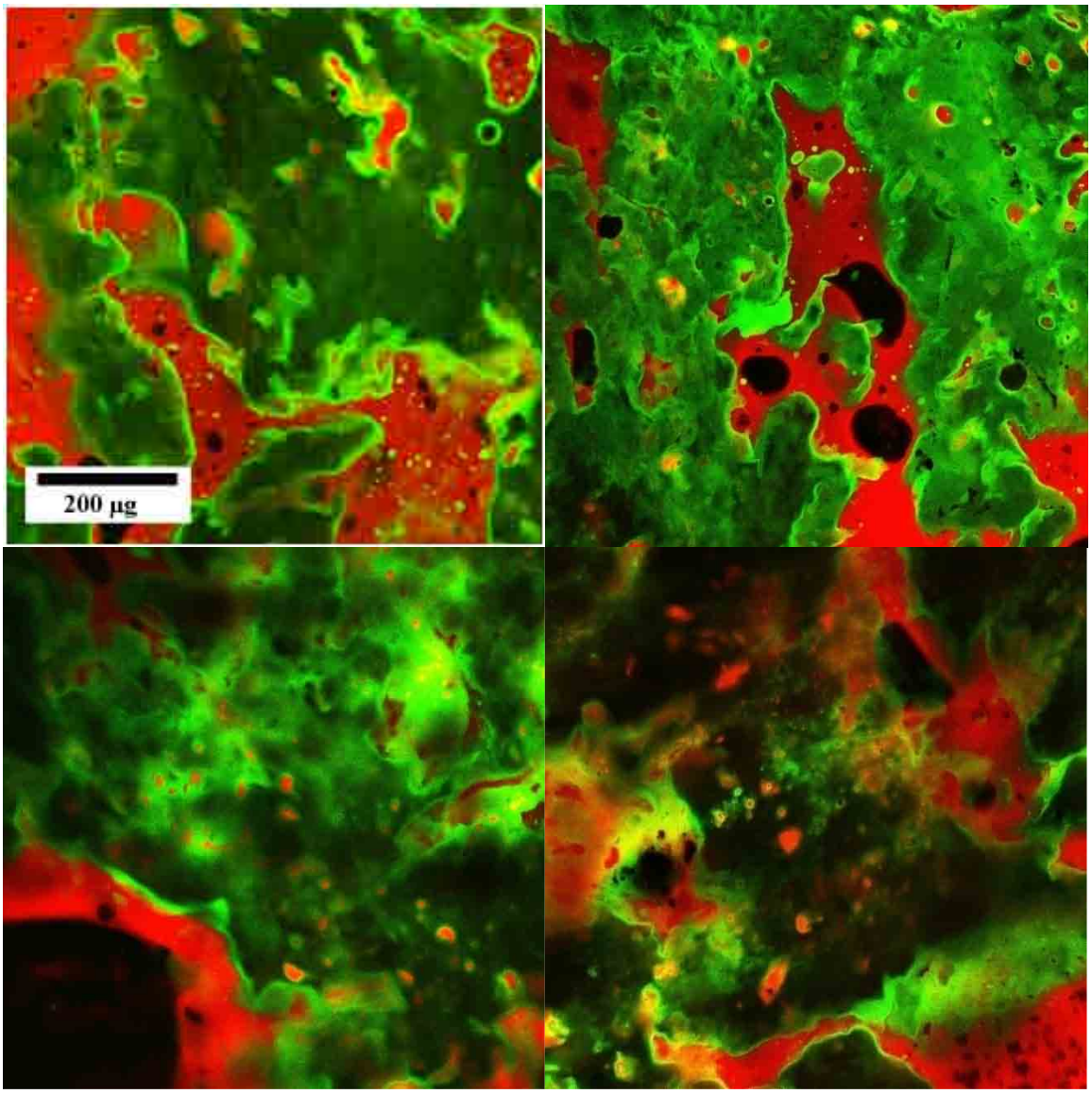

Figure 10. Confocal laser scanning micrographs at d 34 of a high protein (35%) nutrition bar formulated using HWPI with HFCS as a carbohydrate source. Micrographs show components separating into a fat phase and an aqueous phase containing protein and sugar which has large dark areas within it which may indicate sugar separating away from the protein phase. . Green corresponds with dyed protein, red is dyed fat, and black areas are assumed to be the carbohydrate as there is no other un-dyed ingredient in bar.

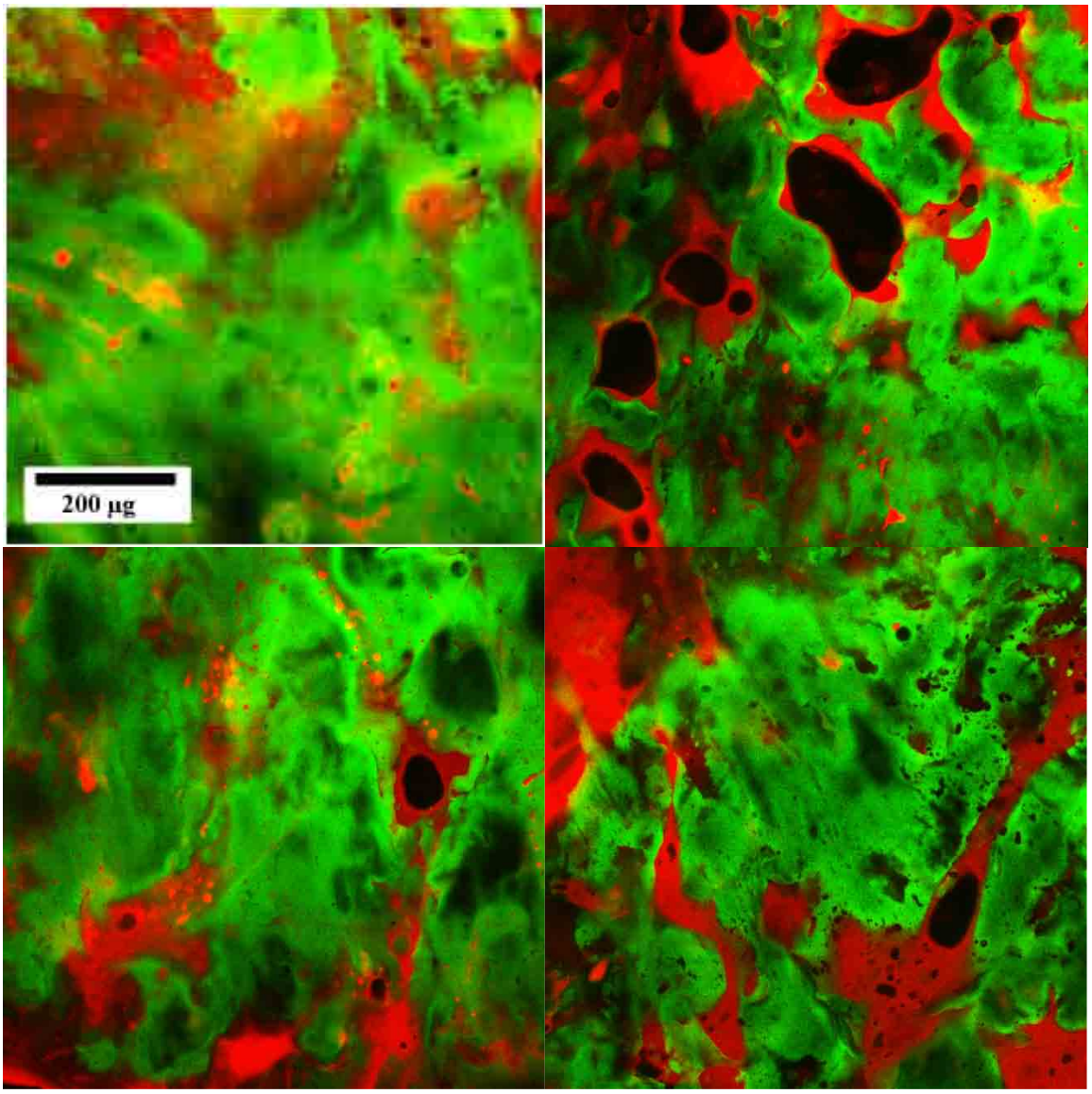

Figure 11. Confocal laser scanning micrographs at d 34 of a high protein (35%) nutrition bar formulated using HWPI with sorbitol as a carbohydrate source. Micrographs show components separating into a fat phase and an aqueous phase containing protein and sugar which is a fairly uniform bright green. Green corresponds with dyed protein, red is dyed fat, and black areas are assumed to be the carbohydrate as there is no other un-dyed ingredient in bar.

Table 6. Percentages of red, green, and black in micrographs of high protein (35%) nutrition bars formulated using either a whey protein isolate (WPI) or a partially hydrolyzed whey protein isolate (HWPI) as a protein source and either high fructose corn syrup (HFCS) or sorbitol as a carbohydrate source after 28 and 34 d of accelerated storage at 32 °C. Red corresponds with dyed fat, green is dyed protein, and black areas are assumed to be the carbohydrate as there is no other un-dyed ingredient in bar.

Storage Time (d)	Treatment											
	WPI/HFCS			WPI/Sorbitol			HWPI/HFCS			HWPI/Sorbitol		
	Red	Green	Black	Red	Green	Black	Red	Green	Black	Red	Green	Black
	----------%----------											
28	3^{az}	28^{az}	65^{az}	5^{az}	24^{az}	68^{az}	18^{bz}	34^{bz}	47^{bz}	18^{bz}	42^{bz}	38^{cz}
34	6^{az}	28^{az}	63^{az}	7^{az}	30^{az}	61^{az}	10^{bz}	53^{ay}	34^{by}	17^{bz}	64^{ay}	18^{cy}

a,b,c Means for the same color within the same row with no common superscript differ ($P < 0.05$).
x,y,z Means within the same column with no common superscript diiffer ($P < 0.05$).

DISCUSSION

Carbohydrate Interactions

The protein and carbohydrate in HPN bars have similar effects on the HPN bars but the magnitude of those effects is distinctly different. Both ingredients may act to inhibit bar hardening through retardation of phase separation, but the effect of the protein is considerably larger than the effect of the carbohydrate on bar hardening. Sugars seems to have only a small effect on the degree of phase separation related to bar hardness and this effect is overshadowed within the exceedingly hard bars as all three ingredients are almost completely phase separated. In those bars that remain soft after storage however, it appears that the sorbitol acts to inhibit phase separation slightly more than HFCS. This is made apparent by the darkening of the carbohydrate/protein phase shown in the HWPI/HFCS bars where the beginnings of a phase separation can be observed as the carbohydrate containing no protein begins to block some of the fluorescence given off by the FITC. Whereas the microstructure of the HWPI/Sorbitol bars shows a very bright green fluorescence throughout the carbohydrate/protein phase that suggests an evenly dispersed protein within the continuous phase of the sugar syrup. Conceivably the sorbitol may function by having a higher hygroscopicity than the sugars in HFCS (Kearsley and Deis 2006). This would allow sorbitol to resist moisture gain or loss as the environmental humidity changes keeping the proteins from becoming fully hydrated which may permit the carbohydrate/protein dispersion to be more energetically favorable. The less acidic pH of sorbitol versus that of HFCS (not measured, based on manufacturer's certificate of analysis for these sugars) might be another means by which the sorbitol inhibits phase separation by electrostatically stabilizing the aqueous phase

permitting the proteins to remain miscible. The presence of a stable alcohol in place of the reactive aldehyde, which makes sorbitol a non-reducing sugar (Kearsley and Deis 2006), may be another way that sorbitol hinders bar hardening as it does not take part in Maillard reactions. Whether or not the differences between the bars containing HFCS and sorbitol are due to the sorbitol being a non-reducing sugar or if it is due to other chemical properties of sorbitol cannot be determined at this time and further research needs to be performed.

Industrial Application

The lack of knowledge about the mechanisms and changes involved in bar hardening has been a detriment to bar manufacturers as it has limited their options when dealing with this problem. As discussed in Chapter 2, hydrolyzed protein has been an effective method for dealing with bar hardening but there may be better solutions. The knowledge that using different sugars in HPN bars can slightly effect the final bar hardness and that sorbitol acts to slightly inhibit phase separation yielding softer bars supports the findings in Chapter 2 that degree of phase separation correlated with bar hardness. This may allow the industry to find other ways to stop phase separation and keep bars soft during storage.

CONCLUSIONS

In HPN bars the amount of hydrolyzed protein has a much greater effect, than the type of sugar, on bar hardness and degree of phase separation. In terms of hardness the order was WPI/HFCS>WPI/Sorbitol>HWPI/HFCS>HWPI/Sorbitol going hardest to softest. In terms of phase separation the order was very similar WPI/HFCS and

WPI/Sorbitol > HWPI/HFCS > HWPI/Sorbitol going from the largest degree of phase separation to least degree. The differences were much smaller however within the protein types as the effect of the sugar type on the phase separation was not observed in the harder WPI bars. In terms of the amount of browning the order was much different with the HWPI/HFCS > WPI/HFCS >> HWPI/Sorbitol and WPI/Sorbitol going most brown to least. The slight decreases in bar hardness and phase separation in the bars containing sorbitol may be related to either the presence of the sorbitol or the lack of Maillard browning and more work will have to be performed to make the determination.

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CHAPTER 4

GENERAL SUMMARY

High protein nutrition (HPN) bars are a complex food system. At manufacture HPN bars are a dispersed system with fat droplets and protein particles dispersed throughout a carbohydrate syrup continuous phase. There is a low amount of water and the protein hydrate slowly as they mix with the carbohydrate syrup to form a two component aqueous phase. It is the degree of phase separation that takes place within this phase that has a significant influence on the hardness of those bars after storage. This is important because hardening of bars during storage is a notable obstacle in the nutrition bar industry and protein and carbohydrates make up ≈ 80% (wt/wt) of most HPN bars.

The type of protein used in HPN bars, specifically whey protein isolate (WPI) or partially hydrolyzed whey protein isolates (HWPI), has a significant effect on the rate of bar hardening. When intact WPI is used in bar formulations the bars go very hard after storage and a three phase separation of the fat, protein, and carbohydrates is observed. In contrast when HWPI is formulated into HPN bars the smaller peptides in HWPI allow the protein phase and the carbohydrate phase to remain miscible throughout storage, and softer bars result. The hydrolyzed protein may accomplish this by having higher water binding peptides that would prohibit water loss, smaller molecular weight peptides which would reduce steric interference between the sugar and the protein, or an increased number of ionizable sites due to the protein hydrolysis may allow for electrostatic stabilization of the sugar/protein aqueous phase. Any of these characteristics inherent in the HWPI could explain their function in keeping bars soft.

The type of sugar used in bars, specifically high fructose corn syrup and sorbitol, has an effect on HPN bar hardness that is much smaller than that of the type of protein. Including sorbitol in the bar formulation will yield softer bars with a smaller degree of carbohydrate/protein phase separation than if HFCS is added. This inhibitory effect on phase separation only noticeable in bars formulated with HWPI as bars containing WPI have a large degree of phase separation regardless of the sugar used which eclipses the effect of the sugar. Sorbitol also is unable to participate in Maillard reactions due to the presence of a stable alcohol in place of an aldehyde. Bars with a smaller degree of Maillard browning also showed a smaller degree of phase separation and bar hardness, but this observation was eclipsed by the large effect of HWPI on bar hardness and phase separation. It is unclear from the data acquired from these studies whether the degree of Maillard browning or just the presence of a different sugar was the cause of the small decrease in hardness and phase separation that was exhibit by the sorbitol.

A very soft bar can be manufactured through the addition of HWPI to the bar formulations due to the inhibition of phase separation by the HWPI. The addition of sorbitol to this formulation would yield a bar that was even softer. These might not be the best solutions to the bars hardening problem however, and with the knowledge that phase separation of the protein from the sugar effect hardness better solution may be developed that might cost less, taste better, or be easier to formulate.

APPENDICES

APPENDIX A: ANOVA TABLES

Table 7. ANOVA of dependent variables for high protein nutrition bar hardness values from experiment 1 outlined in Chapter 2. High protein nutrition bars were formulated so that the protein portion consisted of 0, 25, 50, 75, and 100% partially hydrolyzed whey protein isolate over 36 d accelerated storage at 32 °C.

Sources of Variance	Degrees of Freedom	Mean Square	MSE	F-Value	*P*-value
Amount of HWPI	4	2.89265379	0.012	248.19	<0.0001
Storage Time	5	9.86190597	0.012	846.14	< 0.0001
Amount of HWPI * Time	20	0.14358642	0.012	12.32	< 0.0001

Table 8. ANOVA of dependent variables for high protein nutrition bar percentage of red values from experiment 1 outlined in Chapter 2. High protein nutrition bars were formulated so that the protein portion consisted of 0, 25, 50, 75, and 100% partially hydrolyzed whey protein isolate over 36 d accelerated storage at 32 °C.

Sources of Variance	Degrees of Freedom	Mean Square	MSE	F-Value	*P*-value
Amount of HWPI	4	2.92233788	0.149	19.62	<.0001
Storage Time	4	0.55628983	0.149	3.73	0.0062
Amount of HWPI * Time	16	0.36275664	0.149	2.44	0.0025

Table 9. ANOVA of dependent variables for high protein nutrition bar percentage of green values from experiment 1 outlined in Chapter 2. High protein nutrition bars were formulated so that the protein portion consisted of 0, 25, 50, 75, and 100% partially hydrolyzed whey protein isolate over 36 d accelerated storage at 32 °C.

Sources of Variance	Degrees of Freedom	Mean Square	MSE	F-Value	*P*-value
Amount of HWPI	4	1.28730439	0.079	16.33	<.0001
Storage Time	4	0.30472877	0.079	3.87	0.0050
Amount of HWPI * Time	16	0.16610254	0.079	2.11	0.0102

Table 10. ANOVA of dependent variables for high protein nutrition bar percentage of black values from experiment 1 outlined in Chapter 2. High protein nutrition bars were formulated so that the protein portion consisted of 0, 25, 50, 75, and 100% partially hydrolyzed whey protein isolate over 36 d accelerated storage at 32 °C.

Sources of Variance	Degrees of Freedom	Mean Square	MSE	F-Value	*P*-value
Amount of HWPI	4	21.56655475	0.360	59.94	<.0001
Storage Time	4	2.49995088	0.360	6.95	<.0001
Amount of HWPI * Time	16	0.94346259	0.360	2.62	0.0011

Table 11. ANOVA of dependent variables for high protein nutrition bar water activity values from experiment 2 outlined in Chapter 3. High protein nutrition bars were formulated so that the protein portion consisted of either whey protein isolate (WPI) or a partially hydrolyzed whey protein isolate (HWPI) and was sweetened with either high fructose corn syrup (HFCS) or sorbitol.

Sources of Variance	Degrees of Freedom	Mean Square	MSE	F-Value	*P*-value
Protein Type	1	0.05213522	0.0002	249.70	<.0001
Sugar Type	1	0.00625118	0.0002	29.94	<.0001
Protein Type*Sugar Type	1	0.00208492	0.0002	9.99	0.0042
Day	5	0.00758697	0.0002	36.34	<.0001
Protein Type*Day	5	0.00068405	0.0002	3.28	0.0214
Sugar Type*Day	5	0.00074887	0.0002	3.59	0.0146
Protein Type*Sugar Type*Day	5	0.00015407	0.0002	0.74	0.6024

Table 12. ANOVA of dependent variables for high protein nutrition bar L* values from experiment 2 outlined in Chapter 3. High protein nutrition bars were formulated so that the protein portion consisted of either whey protein isolate (WPI) or a partially hydrolyzed whey protein isolate (HWPI) and was sweetened with either high fructose corn syrup (HFCS) or sorbitol.

Sources of Variance	Degrees of Freedom	Mean Square	MSE	F-Value	*P*-value
Protein Type	1	0.85142609	0.0005	1488.89	<.0001
Sugar Type	1	0.81685742	0.0005	1428.44	<.0001
Protein Type*Sugar Type	1	0.19176304	0.0005	335.34	<.0001
Day	5	0.11417470	0.0005	199.66	<.0001
Protein Type*Day	5	0.01826349	0.0005	31.94	<.0001
Sugar Type*Day	5	0.05943450	0.0005	103.93	<.0001
Protein Type*Sugar Type*Day	5	0.01351967	0.0005	23.64	<.0001

Table 13. ANOVA of dependent variables for high protein nutrition bar a* values from experiment 2 outlined in Chapter 3. High protein nutrition bars were formulated so that the protein portion consisted of either whey protein isolate (WPI) or a partially hydrolyzed whey protein isolate (HWPI) and was sweetened with either high fructose corn syrup (HFCS) or sorbitol.

Sources of Variance	Degrees of Freedom	Mean Square	MSE	F-Value	*P*-value
Protein Type	1	4.55131535	0.131	34.68	<.0001
Sugar Type	1	82.39726975	0.131	627.77	<.0001
Protein Type*Sugar Type	1	0.00000000	0.131	0.00	1.0000
Day	5	2.19285056	0.131	16.71	<.0001
Protein Type*Day	5	2.63186422	0.131	20.05	<.0001
Sugar Type*Day	4	5.01215946	0.131	38.19	<.0001
Protein Type*Sugar Type*Day	1	0.00000000	0.131	0.00	1.0000

Table 14. ANOVA of dependent variables for high protein nutrition bar b* values from experiment 2 outlined in Chapter 3. High protein nutrition bars were formulated so that the protein portion consisted of either whey protein isolate (WPI) or a partially hydrolyzed whey protein isolate (HWPI) and was sweetened with either high fructose corn syrup (HFCS) or sorbitol.

Sources of Variance	Degrees of Freedom	Mean Square	MSE	F-Value	*P*-value
Protein Type	1	0.85142609	0.006	1488.89	<.0001
Sugar Type	1	0.81685742	0.006	1428.44	<.0001
Protein Type*Sugar Type	1	0.19176304	0.006	335.34	<.0001
Day	5	0.11417470	0.006	199.66	<.0001
Protein Type*Day	5	0.01826349	0.006	31.94	<.0001
Sugar Type*Day	5	0.05943450	0.006	103.93	<.0001
Protein Type*Sugar Type*Day	5	0.01351967	0.006	23.64	<.0001

Table 15. ANOVA of variance sources for high protein nutrition bar hardness values from experiment 2 outlined in Chapter 3. High protein nutrition bars were formulated so that the protein portion consisted of either whey protein isolate (WPI) or a partially hydrolyzed whey protein isolate (HWPI) and was sweetened with either high fructose corn syrup (HFCS) or sorbitol.

Sources of Variance	Degrees of Freedom	Mean Square	MSE	F-Value	*P*-value
Protein Type	1	57.02729232	0.065	876.28	<.0001
Sugar Type	1	2.70360583	0.065	41.54	<.0001
Protein Type*Sugar Type	1	0.14496763	0.065	2.23	0.1486
Day	5	2.50096014	0.065	38.43	<.0001
Protein Type*Day	5	0.51416329	0.065	7.90	0.0002
Sugar Type*Day	5	0.18914890	0.065	2.91	0.0344
Protein Type*Sugar Type*Day	5	0.07131022	0.065	1.10	0.3884

Table 16. ANOVA of dependent variables for high protein nutrition bar percentage of red values from experiment 2 outlined in Chapter 3. High protein nutrition bars were formulated so that the protein portion consisted of either whey protein isolate (WPI) or a partially hydrolyzed whey protein isolate (HWPI) and was sweetened with either high fructose corn syrup (HFCS) or sorbitol.

Sources of Variance	Degrees of Freedom	Mean Square	MSE	F-Value	*P*-value
Protein Type	1	25.91247155	0.243	106.75	<.0001
Sugar Type	1	1.60968404	0.243	6.63	0.0117
Protein Type*Sugar Type	1	0.00132901	0.243	0.01	0.9412
Day	1	0.00027546	0.243	0.00	0.9732
Protein Type*Day	1	4.14208321	0.243	17.06	<.0001
Sugar Type*Day	1	0.18189311	0.243	0.75	0.3890
Protein Type*Sugar Type*Day	1	0.75284749	0.243	3.10	0.0817

Table 17. ANOVA of dependent variables for high protein nutrition bar percentage of green values from experiment 2 outlined in Chapter 3. High protein nutrition bars were formulated so that the protein portion consisted of either whey protein isolate (WPI) or a partially hydrolyzed whey protein isolate (HWPI) and was sweetened with either high fructose corn syrup (HFCS) or sorbitol.

Sources of Variance	Degrees of Freedom	Mean Square	MSE	F-Value	*P*-value
Protein Type	1	6.87923324	0.055	125.02	<.0001
Sugar Type	1	0.17417794	0.055	3.17	0.0787
Protein Type*Sugar Type	1	0.42450756	0.055	7.71	0.0067
Day	1	1.77970100	0.055	32.34	<.0001
Protein Type*Day	1	0.76689856	0.055	13.94	0.0003
Sugar Type*Day	1	0.04690341	0.055	0.85	0.3584
Protein Type*Sugar Type*Day	1	0.10623626	0.055	1.93	0.1682

Table 18. ANOVA of dependent variables for high protein nutrition bar percentage of black values from experiment 2 outlined in Chapter 3. High protein nutrition bars were formulated so that the protein portion consisted of either whey protein isolate (WPI) or a partially hydrolyzed whey protein isolate (HWPI) and was sweetened with either high fructose corn syrup (HFCS) or sorbitol.

Sources of Variance	Degrees of Freedom	Mean Square	MSE	F-Value	*P*-value
Protein Type	1	13.39231535	0.079	169.25	<.0001
Sugar Type	1	0.87405348	0.079	11.05	0.0013
Protein Type*Sugar Type	1	0.94725124	0.079	11.97	0.0008
Day	1	2.44849723	0.079	30.94	<.0001
Protein Type*Day	1	1.38851221	0.079	17.55	<.0001
Sugar Type*Day	1	0.30789144	0.079	3.89	0.0517
Protein Type*Sugar Type*Day	1	0.13410201	0.079	1.69	0.1964